NASA CR-54077
376/7014/TII

FINAL REPORT
14 March 1963 - 13 March 1964

RESEARCH ON APPLIED BIOELECTROCHEMISTRY

by

J. H. Canfield and B. H. Goldner

prepared for

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

May 8, 1964

CONTRACT NO. NASw-623

Technical Management
NASA Lewis Research Center
Cleveland, Ohio
Space Electric Power Office
Meyer R. Unger

MAGNA CORPORATION
1001 South East Street
Anaheim, California

NOTICE

This report was prepared as an account of Government-sponsored work. Neither the United States, nor the National Aeronautics and Space Administration (NASA) nor any person acting on behalf of NASA:

a. Makes any warranty or representation expressed or implied with respect to the accuracy, completeness, or usefulness of the information contained in this report, or that the use of any information, apparatus, method, or process disclosed in this report may not infringe privately-owned rights; or

b. Assumes any liabilities with respect to the use of, or for damages resulting from the use of any information, apparatus, method, or process disclosed in this report.

As used above, "person acting on behalf of NASA" includes any employee or contractor of NASA, or employee of such contractor, to the extent that such employees or contractor of NASA, or employee of such contractor prepares, disseminates, or provides access to, any information pursuant to his employment or contract with NASA, or his employment with such contractor.

Requests for copies of this report should be referred to:

National Aeronautics and Space Administration
Office of Scientific and Technical Information
Washington, D. C. 20546

Attention: AFSS-A

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

SUMMARY

A study of bioelectrochemical methods of utilizing human wastes for life support applications in space was conducted. Microbiological and enzymatic conversion of urine and feces to electroactive substances was used as an approach to produce electrical energy. Conversion of urea to ammonia and fecal polysaccharides to hydrogen was investigated. Bacillus pasteurii, selected on the basis of relative ureolytic activity in urine, produced approximately 0.5 mg of ammonia per ml of culture per hour during steady state growth in continuous culture. Electrochemical performance was poor however; ammonia apparently poisoned the platinized platonium anode. Neither mixed microbial cultures nor pure cultures of Escherichia coli grown in feces yielded significant quantities of hydrogen. Hydrolysis of complex fecal components by enzymatic pretreatment with cellulase and lipase was also utilized.

Methods of localizing bacteria at electrodes to improve electrochemical performance were investigated with some success in preliminary experiments.

Studies were initiated, but not completed, to determine the feasibility of electrochemically poising the E_h of activated sludge for greater efficiency in conversion of wastes to reusable chemicals.

It was generally concluded that the amount of power that could be derived from human wastes by this approach was insufficient to contribute significantly to the requirements of space vehicles.

1. INTRODUCTION

The purpose of work undertaken during Contract NASw-623 was to find bioelectrochemical methods for the utilization of human wastes (urine and feces) in space vehicles. The principal function of this program was to obtain power from bioelectrochemical cells using human wastes. A secondary function of this program was bioelectrochemical conversion of the waste materials to chemicals that can be reused in the maintenance of the space vehicle and its occupants.

This effort has been subdivided into three major tasks. The first consisted of a literature search and preliminary selection of organisms and enzymes which are attractive (a) for production of electrochemically active chemicals from human wastes and (b) for conversion of waste materials. The experimental effort was divided into a biological task and an electrochemical task. The biological task was concerned with screening and characterization of microorganisms and enzymes with respect to the most effective conversion of wastes to electroactive chemicals and to otherwise useful chemicals. The electrochemical task was concerned with the evaluation of biological electrodes with respect to the effective utilization of the waste materials, primarily for the production of power. A further function of the electrochemical task was to explore means whereby bioelectrochemical methods might contribute to the conversion of wastes to usable chemicals.

Other programs which relate directly to the present work are being undertaken by Marquardt Corporation under Contract NASw-654 and Philco Corporation, Aeronutronic Division, under Contract NASw-655. These programs are concerned with development and fundamental research in bioelectrochemistry of human wastes, respectively. Magna Corporation, under Contract DA 36-039 SC-90866, is presently performing research on biochemical fuel cells. Further, Magna recently terminated a contract with the Department of the Navy (NObs 84243) during which biochemically promoted power sources were studied.

The authors wish to acknowledge the valuable technical contributions of C. Albright, J. Cavallo, J. Dittman, M. Lechtman and Dr. R. Lutwack. Drs. C. Golueke and W. Oswald served as able consultants to the project.

RESEARCH ON APPLIED BIOELECTROCHEMISTRY

by

J.H. Canfield and B.H. Goldner

ABSTRACT

Human urine and feces were investigated as sources of electrochemical fuels resulting from degradative microbial and enzymatic processes. Bacillus pasteurii was selected to produce ammonia from urinary urea and Escherichia coli and a mixed sewage culture was selected to yield hydrogen from fecal polysaccharides. Cellulase and lipase were considered for enzymatic pretreatment of feces to facilitate subsequent microbial action.

Studies were performed under static and continuous flow conditions to evaluate electrochemical performance of ammonia produced by B. pasteurii.

3. CONFERENCES

A conference attended by personnel of Magna Corporation and Marquardt Corporation was held at Marquardt on June 10, 1963. The purpose of this conference was the discussion of (1) the two organizations' current efforts in bioelectrochemistry relating to Contracts NASw-623 and NASw-654, (2) coordination of the two efforts to avoid possibilities of duplication of effort, and (3) coordination of the two contractors' efforts in obtaining consistent starting materials (human feces and urine).

It was concluded that duplication of effort could most readily be avoided by Magna's efforts being directed primarily to pure cultures of microorganisms, or relatively well defined mixtures of organisms, and isolated enzymes, and by Marquardt's efforts being directed primarily to indigenous organisms of feces.

Conferences were held at approximately monthly intervals between Magna Corp. and Marquardt Corp. personnel during most of the contract period. The purpose of these conferences was the coordination of efforts relating to Contracts NASw-623 and NASw-654. Duly July, 1963, personnel of Philco Corp., working on their Contract NASw-655, visited Magna Corp. facilities to discuss mutual problems relating to their respective contracts. During the same month, Magna Corp. personnel visited Philco facilities for the same purpose.

A meeting was held at Magna Corp. on August 2, 1963 and attended by Dr. M. G. Del Duca, Dr. E. Cohn, H. Schwartz and P. Pomerantz, all of NASA, to discuss technical status of Contract NASw-623. A similar meeting was held at Magna Corp. on September 12, 1963, attended by M. Unger and P. Pomerantz of NASA. An important question discussed at these two meetings, but only partially resolved, was the emphasis to be placed on work under the program on power generation vs. waste handling aspects. Mr. Unger proposed a meeting of all concerned with the contract to be held later in the year at which, among other business, this question would be resolved.

A conference was held at Magna Corp. on December 4, 1963 and was attended by Dr. E. Cohn and M. Unger of NASA, to review the technical status of Contract NASw-623. It was concluded that degradation of human feces and urine in biochemical fuel cells do not produce significant power or substantially reduce the power requirements of a waste management system. Due to the short time remaining in the contract, it was decided to terminate work oriented at deriving power from feces in favor of emphasizing utilization of urine for bioelectrochemical conversion to chemicals useful in a waste management system, and the simultaneous production of power.

4. FACTUAL DATA

4.1 Introduction

A search of the literature pertinent to the microbiology and enzymology of human feces and urine, and primarily directed to the utilization of these materials as electrochemical fuels in space vehicles, was the first step in the research program. The literature search is reported herein in its entirety.

Subsequent to searching the literature, an experimental program was formulated which was directed to qualification of optimum biological conditions and optimum electrochemical conditions for power generation based on the conversion of urine and feces to known electrochemically active intermediates. The secondary problem, conversion of wastes to chemicals which are reusable in a closed environment, was approached via application of electrochemical means to improve the efficiency of aerobic waste degradation.

4.2 Literature Search

It was found convenient to subdivide the literature search into a number of categories: (1) fecal components, (2) urine components, (3) biological waste treatment, (4) microbial production of ammonia and hydrogen, and (5) enzymes. Preliminary evaluation of the literature revealed that very little information was available on biological conversion of human feces or urine *per se*, but that the most nearly related data existed in the literature on sewage treatment. In the absence of specific information on feces and urine, information was sought on components of these materials to guide the search of the microbiological and enzymological literature. An early decision was made to consider only those enzymes which would provide relatively extensive breakdown of human wastes. Thus only hydrolytic enzymes were searched.

In the interest of optimizing power production from waste materials, the literature was searched for information on microbial production of hydrogen and ammonia, considered to be the most likely electro-active chemicals which could be obtained in significant quantities from urine and feces.

4.2.1 Human Fecal Components

The average adult produces 80 to 150 grams of feces daily, which contains a wide variety of organic and inorganic materials. Feces generally contain 65 to 75% water; approximately 25 to 50% of the solids are composed of bacteria, both living and non-living. The major fecal flora of man (excluding protozoa) is presented in Table 1. Other fecal components include mucous, digestive fluids, undigested food, indole skatole, hydrogen sulfide, methyl-mercapton, methane, hydrogen, carbon dioxide and ammonia.

The polysaccharide content consists largely of cellulose, vegetable fibers and pentosans. Low molecular weight saccharides are completely digested and therefore do not appear in feces.

The protein content is primarily of bacterial origin with some contribution from food residues, intestinal cells and free enzymes.

Lipids represent the largest single organic fraction, about 3% of the total weight. The lipid content is composed largely of sterols with individual fatty acids of palmitic, stearic, oleic, myristic, lauric, linoleic, and isomers of oleic acid.

The caloric content of feces is low, ranging from 70 to 140 kilo-calories per man-day. This is in contrast with a daily intake of 2,000 - 3,000 calories.

The most important components of feces are compiled in Table 2. Note that only a small portion of the total constituents of feces have been identified.

TABLE I

The Fecal Flora of Man

Microorganism	Reference
Bacteroides melaninogenicus	Zubrzycki and Spaulding (1)
Bacteroide sp.	Smith and Crabb (2)
Escherichia coli	Zubrzycki and Spaulding (1)
	Smith and Crabb (2)
	Buthaux and Mossel (3)
Aerobacter aerogenes	Zubrzycki and Spaulding (1)
	Buthaux and Mossel (3)
Lactobacillus sp.	Zubrzycki and Spaulding (1)
	Smith and Crabb (2)
Streptococcus sp.	Zubrzycki and Spaulding (1)
	Buthaux and Mossel (3)
Streptococcus faecalis	Smith and Crabb (2)
Streptococcus faecum	Smith and Crabb (2)
Streptococcus liquefaciens	Smith and Crabb (2)
Diphtheroids	Zubrzycki and Spaulding (1)
Clostridium sp.	Zubrzycki and Spaulding (1)
Clostridium welchii	Smith and Crabb (2)
	Buthaux and Mossel (3)
	Collee, Knowlden and Hobbs (4)
Bacillus sp.	Zubrzycki and Spaulding (1)
Pseudomonas sp.	Zubrzycki and Spaulding (1)
Pseudomonas fluorescens	Brisou (5)
Proteus sp.	Zubrzycki and Spaulding (1)
Staphylococcus sp.	Zubrzycki and Spaulding (1)
Staphylococcus aureus	Smith and Crabb (2)
Yeast sp.	Zubrzycki and Spaulding (1)
Treponema dentium	Rosebury (6)
Borrelia refringens	Rosebury (6)

TABLE 2

Proximate Composition of Feces (7)

Component	Weight (g)	Percent of Total
Bulk	150	
Water	99	66.0
Dry Matter	27	17.8
Fat	4.7	3.0
Protein	3.2	2.1
Nitrogen	1.5	1.0
Mono and oligosaccharides	not generally found	
Polysaccharides	varies widely with diet	
Minerals	2.1	1.4
Sodium	0.12	
Potassium	0.47	
Calcium	0.64	
Magnesium	0.20	
Chloride	0.09	
Phosphorus	0.51	
Sulfur	0.13	
Trace Elements (7,8)		
Strontium	0.590 mg	
Copper	1.020 mg	
Iron	28.800 mg	
Lead	?	
Lithium	2.600 mg	
Manganese	3.430 mg	
Nickel	2.900 mg	
Cobalt	1.400 mg	
Zinc	?	
Arsenic	?	
B Vitamins (9)	0.015	0.01
Bile pigments	0.15	0.1

4.2.2 Human Urine Components

Urine is better characterized than feces. Detailed lists of urinary components have been reported,(10,11,12) but the major constituents in an average 24-hour output are water, 1200 g, urea, 22.0 g, chloride (as sodium chloride), 12.0 g, sodium, 4.0 g, sulfur (as sulfur dioxide), 2.5 g, amino acids, 2.5 g, inorganic sulfates (as sulfur trioxide), 2.0 g, potassium, 2.0 g, hippuric acid, 1.5 g, and creatinine, 1.5 g.

4.2.3 Biological Waste Treatment

The maintenance of a closed ecology requires the recovery of all human wastes and the conversion of these materials to food, oxygen, and potable water. The objective of waste-recovery processes, whether physical, chemical, or biological, is to recover as much of the waste materials in a usable form as possible. Ideally, the waste process should convert waste materials into products which can be used in the food production system or recover the wastes in a form which can be directly used by man. Therefore, water and some inorganic compounds may be recovered and returned directly to man. Organic material may be converted to more oxidized compounds, such a H_2O, CO_2, NO_3^- and used in the food production process.

Although biological processes are considered to be more bulky and less reliable than most physical processes, they have the advantage of maintaining the maximum amount of waste materials in a biologically available form. This would allow the reutilization of the by-products of waste conversion processes directly by man or by any of the various related biological support systems, such as photosynthetic gas exchangers. The extent to which conventional microbiological waste treatment processes can be adapted to space capsule waste handling cannot be fully assessed without the benefit of further research. There is little question, however, that the most logical approach to this problem involves directly applying present waste-treatment technology as the point of departure for closed ecology studies.

Ingram (13) has adequately reviewed the microbiology and processes involved in conventional waste treatment. Rather than needlessly duplicate Ingram's effort, our literature search will describe the salient points concerning conventional waste treatment and concentrate more on the microbiology and degradation of human waste as it pertains to our particular interests.

Biological processes for waste disposal convert the organic components of the waste to cell materials which are then partially oxidized by the metabolic activities of the organisms. The products of this metabolic activity are inorganic oxidation products and a mass of microorganisms which is conventionally known as sludge. There are two basic types of biological processes for waste disposal, one aerobic (activated sludge) and the other anaerobic.

In general, there are two groups of bacteria involved in aerobic waste disposal systems. The first group of bacteria utilizes the organic materials in waste and the second group feeds upon their metabolic by-products. This process requires good aeration of the waste and the formation of a floc which results from the growth of zoogleal organisms (14). The organic materials adhere to the surfaces of the floc, which results in rapid degradation. Examining the microbiology of activated sludge, Butterfield (14) isolated a zoogleal bacterium and named it Zoogloea ramigera. Buck and Keefer (15) isolated a similar organism from activated sludge and reported that it produced significant quantities of ammonia from peptones. McKinney and Weichlein (16) isolated many floc-producing bacteria from activated sludge and concluded that more than one organism was involved in floc formation. They considered the organisms listed in Table 3 to be the most significant in aerobic waste disposal. Russian workers (17) concerned with biochemical purification of industrial and domestic effluents have also studied the microbiological flora of activated sludge, and they have reported the isolation of many organisms cited by McKinney and Weichlein (16).

Kaplovsky (17) has differentiated the anerobic process into three basic stages:

TABLE 3

Floc-Producing Bacteria Isolated from Activated Sludge

Zooglea ramigira
Escherichia coli
Escherichia freundii
Pseudomonas perluria
Pseudomonas ovalis
Alcaligenes faecalis
Alcaligenes metalcaligenes
Bacillus megaterium
Lactobacillus caseii
Neisseria catarrhalis
Aerobacter aerogenes
Flavobacterium solare
Flavobacterium breve
Micrococcus conglomeratas
Micrococcus varians
Achromobacter liquefaciens

1. Intensive acid production
2. Intensive digestion or liquification
3. Intensive digestion and gasification

The major gases produced during gasification are methane and carbon dioxide, with small amounts of hydrogen sulfide and hydrogen. Methane is a reliable index of the efficiency of anaerobic digestion and up to 75% of the total volume of gas may be methane. It has generally been observed that obligate anaerobes do not form a major portion of the microorganisms involved in anaerobic sludge digestion but that facultative anaerobes predominate. The microorganisms found in anaerobic sludge are compiled in Table 4.

One of the problems associated with standard waste disposal systems is the large amount of solids remaining after digestion. Aerobic processes are generally more efficient in reducing solids than anaerobic digestion. Leone (21) has reported on aerobic and subsequent anaerobic human waste treatment processes designed for space systems and has found that the activated sludge system stabilized in several hours with a 20% reduction in initial solids (3.7 g/l). Subsequent processing in an anaerobic digestor reduced total solids an additional 15%, but required a month to do so. Kountz and Forney (22) advanced a theory concerning a two-phase activated sludge system that would result in complete oxidation. Phase I would consist of the assimilation of substrates and Phase II involved the endogenous oxidation of metabolic by-products. Their results showed that non-oxidizable sludge accumulated at the rate of 0.6% of the total weight per day.

Garret (23) reported on control of activated sludge growth by regulated overflow to a settling tank. The mathematics involved are very similar to that which apply to continuous culture of microorganisms, and he reported a consistent growth rate of the floc. The use of continuous culture for aerobic waste disposal has been adapted to an algal growth unit by Golueke, Oswald and McGanky (24). Their system used the activated sludge process to digest raw sewage in order to provide the algae with inorganic nutrients such as ammonia

TABLE 4

Microorganisms Isolated from Anaerobic Sludge

Microorganism	Reference
Bacillus subtilis	Ruckhoft, Kallas and Edwards (18)
Bacillus endorhythmas	Buck, Keefer and Hatch (19)
Escherichia coli	Ruckhoft *et al.* (18)
Aerobacter aerogenes	Ruckhoft *et al.* (18)
Methanobacterium formicicum	Ingram (13)
Methanobacterium omelianski	Ingram (13)
Methanobacterium suboxidans	Ingram (13)
Streptococcus diploides	Buck, Keefer and Hatch (19)
Clostridium bifermentans	Bergey's Manual (2)
Cl. perfringens	Bergey's Manual (2)
Desulfovibrio desulfuricans	Bergey's Manual (2)

and carbon dioxide, while the algae produced oxygen for the growth of the bacteria. They found that after the addition of waste to the culture, the fecal odor disappeared in several hours and eventually was replaced by an actinomyces odor - the odor of rich, damp soil.

The Boeing Company (25) is presently studying an activated sludge process in which the wastes are concentrated several hundred times over that of conventional sewage. The digestor volume has been reduced to 3.0 $ft.^3$ with a 48 hour detention time, to stabilize the wastes of five men.

Ingram (13) developed a space-oriented human waste disposal system based upon the activated sludge process, and found the process most efficient if the organisms and concentration of solids were similar to domestic sewage. In agreement with Moyer, (26) Ingram found the predominating gases liberated to be carbon dioxide and small amounts of ammonia.

Relatively little work has been done with anaerobic digestion of human wastes. Ingram (13) found anaerobic digestion of human wastes difficult to initiate, and poorly digested, extremely foul-smelling sludge resulted.

Pote (27), operating a thermophilic anaerobic digestor, reported better success, but added kitchen scraps to human wastes. Volatile solids were reduced from 67 to 49%. The gas produced was about 70% methane and 30% carbon dioxide.

Attractive features of the aerobic process include (1) the rapidity with which wastes are stabilized, (2) end-products which are generally non-odorous and non-toxic, and (3) almost complete degradation. The chief disadvantages with respect to a closed ecology, are the large consumption of oxygen and the production of carbon dioxide.

The chief advantage of an anaerobic process operating in a closed system is that oxygen is not required. Otherwise, this process is slower, is less efficient in reducing solids and produces more obnoxious and toxic by-products than an aerobic process. Also, most anaerobic digestors operate optimally at 55 - 60°C.

4.2.4 Microbial Production of Ammonia and Hydrogen

It was pointed out earlier that aerobic disposal systems usually evolve carbon dioxide and ammonia while anaerobic systems evolve mainly carbon dioxide and methane. It would appear that most of the hydrogen produced is used by the microorganisms for reductive reactions. In an attempt to determine the gases evolved from untreated raw human feces, Wheaton et al. (28) placed fecal samples into tin cans fitted with pressure gauges and gas sampling ports. Fecal samples produced from 3.5 to 6 ml of gas (at STP) per g within 7 days, under air or argon. Gas chromatographic analyses showed that the major constituents were methane, carbon dioxide and ammonia with very little hydrogen and hydrogen sulfide. These authors also reported a particularly interesting observation concerning two distinguishable types of fecal samples. The first type produced carbon dioxide primarily, with a little hydrogen; the second group produced carbon dioxide and methane.

Melpar, Inc. (29) has evaluated a number of microorganisms for their ability to evolve hydrogen. Their study has shown that of the following bacteria: Escherichia coli, Aerobacter aerogenes, Aerobacter cloacae, Serratia kiliensis, Pseudomonas sp. 64A, E. coli produced the highest yield of hydrogen when grown on glucose or maltose. Aerobacter cloacae was next best when grown on galactose, arabinose or glucose. Aerobacter aerogenes produced a good yield of hydrogen from fructose, glucose, maltose, or lactose. Pseudomonas sp. 64A produced a fairly good yield when grown anaerobically on formate. Later studies showed that Clostridium welchii 6785 produced hydrogen at the rate of 8.2 liters per hour from glucose. (30) The production of hydrogen by enteric organisms grown on glucose is of interest because cellulose hydrolysis results in glucose as the final product.

Gest (31) has reviewed various organisms capable of producing hydrogen and ammonia. Cl. butylicum grown on pyruvate will evolve hydrogen and the reaction is apparently due to a phosphoroclastic split with the formation

of acetyl phosphate and formate. The formate is then split to produce carbon dioxide and hydrogen. Cl. kluyverii produces hydrogen in the oxidation of acetaldehyde to acetate. Clostridial reactions also evolve ammonia; e.g., Cl. tetranomorphum attacks single amino acids, such as glutamate, with the production of ammonia, carbon dioxide, hydrogen and volatile acids. Cl. propionicum also attacks single amino acids, but produces carbon dioxide, ammonia and volatile acids, while hydrogen, though evolved, is used for the reduction of oxidized intermediates. Cl. sporogenes utilizes the Stickland reaction, in which one amino acid is oxidized and another is reduced, with the production of two organic acids and two molecules of ammonia. Stickland (32) reported that only specific compounds could act as hydrogen donors and as hydrogen acceptors. The donors were D-alanine, D-valine, pyruvate, D-leucine, L-phenylalanine, L-aspartate and D-glutamate. The acceptors were glycine, proline, and hydroxyproline. Stickland found that the best combinations for ammonia production by Cl. sporogenes were alanine and proline, valine and hydroxyproline, and leucine and glycine. He also reported that Cl. sporogenes would attack either serine or tyrosine alone, with the production of ammonia.

Nisman, Reynard and Cohen (33) studied the Stickland reaction in a number of bacteria, and showed that the following clostridia were physiologically similar to Cl. sporogenes: Cl. histolyticum, Cl. bifermentans, Cl. butyricum, Cl. acetobutylicum, Cl. flabelliferum, Cl. saprotoxicum and Cl. sordellin. They also found that the Stickland reaction did not occur with the following clostridia and facultative anaerobes: Cl. iodophilum, Cl. saccharobutyricum, Cl. welchii, Staphylococcus aureus, Proteus vulgaris, Klebseilla pneumoniae, and E. coli.

Gest (31) also reported that the methane bacteria produce hydrogen during the formation of methane, but that the hydrogen is used to reduce carbon dioxide. If, however, Methanobacterium formicicum or M. vannielii are grown in an alkaline medium (above pH 8.6), carbon dioxide and hydrogen are produced as the major gases.

It was reported above that a number of bacterial species can produce hydrogen from the degradation products of cellulose, but these organisms generally cannot degrade cellulose itself. Khouvine (34) isolated an anaerobic cellulolytic bacterium, Bacillus cellulosae dissolvens, from human feces, herbivora, and soils. This organism produced hydrogen, carbon dioxide, organic acids, and ethanol from cellulose. Cowles and Rettger (35) reported the isolation of a cellulolytic anaerobe, Clostridium cellulosolvens, from horse feces. This organism fermented cellulose and produced gas, 75% of which was hydrogen, symbiotically with Aerobacter aerogenes, E. coli, or Proteus vulgaris. These authors did not believe that Khouvine's culture was pure, however. Nagliski et al. (36) reported that Clostridium roseum fermented cryptostegia leaves rapidly with the production of a considerable amount of gas, and point out the interesting fact that the organism did not dissolve filter paper cellulose in tryptone broth. Hungate (37) reviewed anaerobic mesophilic cellulolytic bacteria, and reported that although sewage sludge contained several species of cellulolytic bacteria, none were found in raw sludge. Fuller and Norman (38) reported that various aerobic, mesophilic soil bacteria were capable of hydrolyzing cellulose. They isolated and described Pseudomonas ephemerocyanea, Ps. lasia, Ps. erythra, Achromobacter picrum, and Bacillus aporrhoeus. Fuller and Norman (39) also performed various biochemical studies with the above organisms and found that each organism would utilize xylans and various hexosans. In a further study, Fuller and Norman (40) showed that lignin usually inhibited the fermentation of xylans and hexosans by all of the bacteria.

A small group of urea hydrolyzing soil bacteria have been termed "urea bacteria" by Alexander (41) because of their tolerance to high levels of the compound, and their nutritional requirement for it. Bacillus pasteurii and Bacillus freudenreichii are representatives of spore formers in this group, and Micrococcus ureae and Sarcina ureae are representatives of the coccus forms. Wiley and Stokes (42) have reported that B. pasteurii requires ammonia, formed by hydrolysis of urea, not only for growth, but also for development of the

alkaline pH necessary for growth. Gibbons and Doetsch (43) reported that nearly all of the ureolytic bacteria are aerobic or facultative anaerobes, and that very few obligately anaerobic bacteria possess this capability. They reported, however, the isolation and characterization of an obligately anaerobic, ureolytic bacterium, which has been classified as Lactobacillus bifidis var. ureolyticus.

Cooke and Keith (44) isolated Brevibacterium ammoniagenes from feces; this organism produced ammonia from urea both aerobically and anaerobically. Better growth occurred aerobically, however.

4.2.5 Enzymes

The literature was searched for information on those enzymes which could be expected to have relatively broad activity toward urine and feces. The use, for the purposes of this program, of enzymes which can act only on a minor component of human wastes would result in expenditure of considerable effort with little to show for it in terms either of power production from the wastes or degradation of the wastes. Accordingly, enzymes were considered from the general point of view that pretreatment of the wastes would facilitate bacterial action. Thus the saccharolytic enzymes cellulase, pectinesterase and polygalacturonase were considered. A proteinase, papain, was considered to the exclusion of other, more specific proteinases. Urease was considered because urea is the most predominant single component of human wastes. Finally, lipases were considered as a means of aiding in degradation of fecal lipids.

4.2.5.1 Cellulase

Cellulase (cell free) has been isolated from a large number of plants and animals. The list below contains but a few representative samples:

Molluscs

Helix pomatia (snail) (45)

Teredo (shipworm) (46)

Protozoa

Endoplodium neglectum (47)

Arthropods

Termes obesus (termite) (48)

Plants

malt (49)

algae sp. (50)

Fungi

Myrothecium verrucaria (51)

Aspergillus niger (52)

Bacteria

Rumen microorganisms (53)

Pseudomonas fluorescens (54)

The largest body of work concerning the purification of cellulase has been accomplished using the fungi as the source material. This is because cellulase is an exoenzyme in most fungi and can be conveniently obtained from the medium by removing the cells and residual cellulose substrate. The fungus most commonly used is Myrothecium verrucaria. A procedure for obtaining crystalline cellulase from the fungus Irpex lacteus has been reported by Nisizawa. (55)

The ability of the enzyme cellulase to hydrolyze reprecipitated cellulose, soluble derivatives, or highly crystalline native celluloses varies widely between extracts from different sources. However, good evidence has been accumulated (56,57,58,59,60) that, as the crystallinity of the substrate decreases the rate of enzymatic hydrolysis increases.

Most plant cellulases have an optimum pH in the region of 5.0, animal cellulases in the range of 5.0 to 5.5, and bacterial cellulases between 5.8 and 7.0. Optimal pH values vary widely for fungal cellulase over a range from pH 3.0 to pH 8.0; however, most pH values are on the acid side, between pH 4.0 and pH 7.0. (61)

There is very little information regarding the temperature optimum of pure cellulase isolated from plant, animal and bacterial sources. In the case of the fungal cellulases where the more definitive work has been done, it has been shown (assay based on production of reduced sugar) that the maximum temperature varies from 40 to 70°C. (62,63,64,65) The temperatures at which the fungal cellulases start to be inactivated vary from 40°C to as high as 70°C. Below are a few examples.

Source	Temp.	Reference
Trichoderma koningi	70°C	66
Poria vaillanti	60-70°C	63
Aspergillus niger	70°C	67
M. verrucaria	50°C	62

Work with fungal cellulase indicates that the enzyme is generally inhibited by mercury, silver, chromium, lead, and zinc salts, while manganese, cobalt, magnesium, and calcium with phosphate cause stimulation in certain cases. (61) The literature concerning the effect of certain oxidizing and reducing agents on the enzyme are conflicting; however, permanganate and molybdate have been found to be stimulating and bisulfite, dithionite and benzoquinone are inhibitory. (68)

Basu and Whitaker (69) reported that fungal cellulase from M. verrucaria was inhibited by iodoacetate and p-chloromecuribenzoate but that the inhibition could be reversed by the addition of glutathione, crysteine, sodium sulfide or potassium cyanide.

Reese, et. al., (70) using cellulose isolated from various micro-organisms, found cellobiose to be generally inhibitory. There were two exceptions, and in these cases the presence of the cellobiose appeared to be stimulatory. These experiments were carried out at pH 5.0 using carboxymethyl cellulose as a substrate and a 2% solution of cellobiose.

The addition of certain proteins to preparations of cellulase from M. verrucaria (71) were shown to stimulate the cellulolytic activity.

There are many methods for the assay of cellulase, but most are based on one of the following procedures:

1. Increase in reducing sugar (colorimetric or volumetric).
2. Effect on viscosity of soluble cellulose derivatives.
3. Loss in weight of insoluble substrates.
4. Oxygen uptake during the enzymatic oxidation of glucose (glucose oxidase) produced by hydrolysis of the cellulose.
5. Decrease in mechanical properties of fibers or films.
6. Changes in bi-refringence of films.

4.2.5.2 Pectic Enzymes, Pectinesterase and Polygalacturonase

Pectic substance is a group designation for colloidal polysaccharides which contain a large proportion of D-galacturonic acid and methanol and which are widely distributed in the plant kingdom. The pectin molecule has an α-1,4-glycosidic linkage between the pyranose rings of the methyl ester of the D-galacturonic monomer. The pyranose ring occurs mainly in the chair form, corresponding to the most stable configuration of D-galactose. The enzymes involved in the degradation of this pectic substance are pectinesterases and polygalacturonase.

Pectinesterase catalyzes the hydrolysis of the ester bonds of pectic substances to yield the pectic or pectinic acids and methanol. (72) Polygalacturonase catalyzes the hydrolysis of glycosidic bonds between deesterified galacturonide residues.

4.2.5.2.1 Pectinesterase

Pectinesterase (PE) has been found to be widely distributed in plants, molds, and bacteria. However, those sources most often used and reportedly containing considerable quantities of PE are alfalfa (72), tomatoes (73), citrus fruit (74), potato leaves, tobacco, elder (75), and fungi (76). In almost all cases the PE is usually fixed or adsorbed to the solid portions of the plant or fruit.

PE has been shown to be highly specific, saponifying only the methyl ester of pectic substances (77). The PE from the skin of oranges has been reported to hydrolyze only the methyl ester groups adjacent to free carboxyl groups, the hydrolysis proceeding linearly along the pectin molecule as successive methoxy groups split off (78).

The temperature stability of PE varies depending on the source from which it was isolated. Fungal PE is 50% inactivated in 0.1 M NaCl at pH 6.0 for 1 hour at 35°C, whereas tomato PE at 70°C and under otherwise similar conditions, was only 50% inactivated (76). Orange PE was 50% inactivated at pH 7.5 in a borate-acetate extract in 30 minutes (79).

The pH optimum for plant PE lies in the range of pH 7 to 8 at low salt concentrations. However, the fungal PE is markedly different in this respect and has a pH optimum in the range of pH 4.6 to 5.5 (80).

PE is relatively inert to chemical inhibition by cyanide, iodine, formaldehyde, etc. (76) Detergents and soaps are reported to be very effective inhibitors of PE (76).

Cations have been found to increase by many fold the activity of PE in the pH range extending several pH units below neutrality. It has also been shown that divalent cations are more effective than monovalent cations. Because of this fact the explanation for the observed increase in activity can not be based on ionic strength. Although some theories have been advanced to explain this phenomena, none seem to completely fit all the facts (76).

The activity of PE is assayed by measuring the increase of carboxyl groups at constant pH (81), the increase of methanol (73), or by measuring the evolution of carbon dioxide from an $NaHCO_3$ buffer using manometric methods (82). To insure reliable results from any of these methods the cation content of the system must be known. The enzyme activity is expressed in millequivalents of bonds hydrolyzed per minute per millimole of enzyme under established "standard" conditions. These "standard" conditions are, in general, the optimum conditions (pH, temp., etc.) and, therefore, vary slightly with the source of the enzyme.

4.2.5.2.2 Polygalacturonase

Polygalacturonase (PG) is most commonly found in the lower plants such as bacteria and fungi, but seldom in higher plants. In animals it is found only in the snails (80). Isolation of PG often involves separation from PE by ion exchange treatment. Successful isolation of PG has been accomplished from the following organisms:

Organism	Method of Separation	Reference
Aspergillus niger	Partial inactivation; adsorption on Fuller's earth. Chromatography on Al_2O_3 and on filter paper.	83
Penicillium expansum	Precipitation with alcohol	84
Rhizopus tritici		

Most of the work done using purified preparations of PG indicates that PG is very specific (85). Although PG is regarded as a hydrolase, it has not yet been clearly determined whether PG splits the glycosyl-oxygen bond or the aglycone-oxygen bond. Since no transferase action has yet been reported, it may be that a double displacement mechanism as proposed for many carbohydrases is operative (77).

A classification of various PG's has been proposed based on their mode of action. There are three classes (77) :

PG I Liquifying PG - splits the glycosidic linkages more or less at random, producing a rapid decrease in viscosity. This type acts best on pectins of a low degree of esterification.

PG II Brings about the hydrolysis of highly esterified pectins. The commercial preparation "Hydrolase" appears to be just such a PG.

PG III Splits glycosidic linkages from one end of the pectin molecule - probably from the reducing end.

It has been observed that alkali salts increase the activity of PG as they do PE, however, there is not nearly the marked effect for PG. The effect follows the series:

$$Li < Na < K < Rb < Cs \quad (86)$$

In contrast to the effect observed with PE, Mg^{++} caused 48% inhibition of PG (87).

The pH optima for PG have been reported from 3 to 6. Although the optimum may vary depending on the source of the enzyme, pH 4.0 is most

generally used (80). PG's from bacteria and phycomycetes have a pH optimum near 7.0 (77). PG appears to be remarkably stable to acid; it is only 50 to 70% inactivated in 20 minutes at pH 0.6 and 23°C. PG (from Aspergillus niger) appears to be rather thermostable (88,89).

Assay procedure is based primarily on one of the following:

(a) Increase in reducing groups (90).

(b) Decrease in viscosity (91).

(c) Decrease in precipitability by calcium or nonpolar solvents.

(d) Decrease in optical rotation.

The assay procedure most commonly used is that based on the reducing group change. The results can be conveniently expressed in milli-equivalents of bonds hydrolyzed or millimoles of reducing group per minute under standard assay conditions. Standard conditions consist of pH 4.0, 25°C and 0.5% pectic acid.

4.2.5.3 Lipase

The enzyme lipase catalyzes the hydrolysis of oil and fats, i.e., triglycerides of long-chain fatty acids and can be thus differentiate from other esterases which hydrolyze simple esters of low molecular weight acids.

Isolations of the enzyme lipase have been accomplished primarily from the pancreas of various animals, i.e., hog (92,93), rats (94), and bovine pancreas (95). The isolation of lipase has also been reported from fungi (96), yeast (97), castor bean (98), and various bacteria (99,100).

Since it has been shown that lipase makes up 2.5% of the total protein of swine pancreas this has remained the primary sourcefor this enzyme. A recent procedure for the isolation of pancreatic lipase (hog) has been reported(101). This preparation was found to have maximum stability at pH 5.6, and was able

to withstand storage in 5% NaCl at 5°C for 2-3 months without loss of activity. There is some indirect evidence that calcium ion is necessary for stability of the enzyme (102). Hg and other heavy metals and also halogen ions (F > I > Br > Cl) seem to inhibit the enzyme (103). However, it appears that -SH groups are not directly indispensable for the hydrolytic activity. Lipase is inactivated by surface-active compounds (102).

Desnuelle (101) provides evidence that the lipase enzyme is more active when the substrate is emulsified (rather than in solution) and that the enzyme is active at the oil-water interface. He provides a series of curves showing that the initial enzyme rate varies with the interfacial area, and also reports Michaelis curves using partially soluble substrates (triacetin, methyl butyrate) which show a significant increase in lipase activity after the substrate has reached saturation and is in the form of an emulsion.

There is good evidence (104) that lipolysis occurs in three well-separated and consecutive steps and is due to the catalytic effect of one enzyme (lipase). The requirement for calcium ion does not in any way alter the course of the lipolysis but does promote the process by combining with the interfacial soaps (105).

Desnuelle gives evidence that bile salts, such as taurocholate, increases the initial rate of hydrolysis by a factor of four at 37°C. He eliminates the possibility that the acceleration is due to an increase in interfacial area by insuring that it is already large enough to give maximal reaction rate before the addition of taurocholate. Since there are normally some bile salts present in fecal material, one can anticipate some stimulation of the lipase activity when feces serves as the substrate. According to Goldblith and Wick (7), feces contains 0.15% bile pigments. Optimum pH is about pH 7.0 for lower triglycerides; it is shifted to pH 8.8 for higher triglycerides (106). Pancreatic lipase (although it varies with the state of purify of the enzyme, with buffers, with method of assay, etc.) has an optimum temperature of about 37°C for most substrates (107).

Two methods have been used for assay. Continuous titration of fatty acids released during hydrolysis is one of these (108). The other is measurement of carbon dioxide released from sodium bicarbonate by the fatty acids (109).

4.2.5.4 Papain

Papain was chosen over such proteolytic enzymes as pepsin, chymotrypsin and trypsin for the following reasons: (1) the latter enzymes are not as heat stable, (2) they are more sensitive to low concentrations of urea, (3) their optimum pH values are at very acid levels (pH 2-3), (4) papain has shown to produce more extensive degradation of protein substrates than other proteases, and (5) crystalline papain is relatively easy to prepare and is commercially available.

Isolation is from the dried latex of the papaya plant. The dried extract is ground with sand in the presence of a dilute cysteine solution at pH 5.7. Inert material is removed from the clarified extract after the solution is brought to pH 9.0. The enzyme is precipitated first with ammonium sulfate and subsequently with sodium chloride. Crystallization is achieved by allowing a solution of the enzyme at pH 6.5 to remain at 4°C. Recrystallization can be accomplished from sodium chloride solution or from 70% ethanol by salting out with a lithium salt.

One of the most striking properties of papain is its rather high temperature stability: half-life, 56 min. at 75°C (110). Resistance to elevated temperature is markedly pH-dependent, particularly below pH 4.0, where papain is rapidly and irreversibly inactivated (111).

In the presence of protein substrates (as opposed to synthetic peptides) papain was found to be fully active after exposure to 9M urea (101). However, in the presence of synthetic substrates it was found that concentrations about 3M urea effected irreversible inactivation of papain (112).

The enzyme is sensitive to sulfhydryl agents such as iodoacetate, hydrogen peroxide, heavy metals and p-mecuribenzoate, and is activated by H_2S, HCN, and other reducing agents (113,114).

The broad specificity of papain was shown by Calvery (115) who studied the hydrolysis of crystalline egg albumin by crude papain and found that after papain digestion, neither pepsin or trypsin could bring about further hydrolysis. The pH optimum for protein digestion was in the range pH 7.0-7.5 (116).

Papain may be conveniently assayed by measuring the rate of hydrolysis of a synthetic substrate such as benzoyl-L-argininamide (BAA). The assay is performed at pH 5 to 7 at 40°C in the presence of appropriate activating agents (117).

Papain can also be assayed with urea-denatured hemoglobin as substrate (118,119). The reactions are performed at 39°C in the presence of 0.005 M cysteine and 0.001 M EDTA at pH 7 to 8.5 Aliquots of the reaction mixture are removed at appropriate intervals, treated with 5% trichloroacetic acid, and filtered. The filtrates are examined in a spectrophotometer at 280 mμ.

4.2.5.5 Urease

The presence of urease has been reported in numerous bacteria, animals and some plants. However, the two richest sources and those most frequently used for the isolation of the enzyme are jack bean meal (116) and the bacteria B. pasteurii (120). Jack bean contains 0.15% urease (on a dry weight basis) and B. pasteurii 1.0%. Isolation from jack bean meal is fairly straightforward, an acetone extract giving impure crystalline material (121). Isolation from B. pasteurii is much more complex, involving six steps, including several sulfate fractionations, calcium phosphate gel treatment, and an acetone fractionation. Although the enzyme was not obtained in crystalline form,

Larson and Kallio et al. (120) have reported activities of 150 to 190 units/mg compared to 130 units/mg for Sumner's jack bean urease.

The presence of sulfhydryl groups in the urease molecule has been amply confirmed (122, 123). The work in the last reference indicates that there are 23 -SH groups per mole. Due to the presence of the -SH groups, urease is inhibited by typical -SH reagents such as the metal ions Ag^{+}, Hg^{++}, Cu^{++}, ferricyanide, p-mercuribenzoate, trivalent arsenicals, and furacin. Sodium and potassium ions inhibit, and phosphate ions activate urease (124).

Studies using tri-(hydroxymethyl)aminomethane sulfur (inert) as a buffer (125) show a pH optimum of 8.0. Wall and Laidler (126) indicate that at urea concentrations above 0.3 M the activity actually decreases. It has been postulated (126) that at high concentrations, urea may occupy that site on the urease surface normally occupied by water, and thereby inhibit the enzyme.

Extensive studies to determine the heat of activation of urease (127, 128) indicate that thermal deactivation of the enzyme is significant at 40 - 50°C and that only 10% of original activity of the enzyme (at pH 7.0) remained after heating to 96°C for six minutes.

By comparison, the bacterial urease isolated by Larson and Kallio (120) was very sensitive. The reported that their purest preparations (190 units/mg) were inactivated very rapidly on standing (even during refrigeration), by dialysis in the absence of reducing agents, by pH values lower than 5.2, and by organic solvents.

The enzyme is absolutely specific for hydrolysis of urea (129, 130).

A colorimetric assay using the Nessler reagent to determine the ammonia formed from the hydrolysis of the urea is convenient and simple (116).

A titrimetric assay is reported (131) in which the ammonia formed is titrated with 0.1 M HCl.

4.3 Power from Human Wastes

4.3.1 Theoretical Fuel Value

From the heats of combustion of the human wastes, urine and feces, a maximum value can be estimated for power that can be produced using these as fuels. Obviously, various limitations apply; however, the power is to be extracted, and the heats of combustion provide only a limiting value. Heats of combustion are used because the most likely approach to producing power from urine and feces electrochemically will be to employ oxygen as the oxidant.

4.3.1.1 Urine

The major organic constituent of urine is urea. While urea production is dependent on a number of factors, the normal average quantity produced per day by one man is 22 g. Higher than normal urea production is encountered when high protein diets are consumed; up to twice the normal urea output may be obtained under such conditions. Other significant organic materials in urine, again based on an average man-day, are amino acids, 2.5 g, creatinine, 1.5 g, and hippuric acid, 1.5 g. The following tabulation shows the contribution of each of these components to the heat of combustion or urine:

	Kcal	Watt hours
Urea	58	68
Amino acids (taken as 1/2 alanine, 1/2 phenylalanine)	14	16
Creatinine	7.5	8.7
Hippuric acid	8.5	9.9
Total	88	102.6

Note that more than half the heat of combustion derives from the single component urea. Further, the relatively high figure quoted for amino acids is very approximate and would come from many more individual compounds than the two used for the estimate.

The heat of combustion of urine provides a guide to the maximum energy that might be obtained from it. Further consideration must be given to the processes for obtaining such energy and to the limitations which apply to these processes.

Practical considerations indicate that a single process, in terms of the controls that must be applied, would be most desirable for conversion of the chemical energy of urine to electrical energy. Because the components of urine are not yet amenable to direct conversion in power-producing reactions, at least two processes must be considered: a chemical conversion step to produce an electrochemically active intermediate and the electrode reaction process. As presently conceived, both these processes would be combined. That is, the chemical transformation, effected through some form of biological catalysis, would be carried out in the presence of an electrode.

One of the most practical expedients is to concentrate on the most abundant component of urine and to maximize the efficiency of converting the chemical energy of that component to electrical energy. In urine, the most abundant component which can lead to an electrochemically active intermediate is urea.

Earlier, the normal average output of 22 g urea per man-day was equated with approximately 68 watt hours based on heat of combustion data. Let us consider (1) how this heat of combustion can be converted to electrical power, and (2) how much of this energy might be practically obtained. Without considering a mechanism for this conversion (mechanism will be treated subsequently), urea may be hydrolyzed to ammonia and carbon dioxide:

$$H_2NCONH_2 + H_2O \longrightarrow 2\ NH_3 + CO_2$$

Ammonia is an electrochemically active intermediate, which can in turn be employed at a fuel cell anode:

$$2\ NH_3 \longrightarrow N_2 + 6\ H^+ + 6\ e^-$$

Assuming oxygen as the fuel cell oxidant, the following cell reaction may be taken as a model and used for estimating efficiency:

$$2\ NH_3 + 3/2\ O_2 \longrightarrow N_2 + 3\ H_2O, E^\circ = 1.13\ V$$

Assuming that essentially no losses occur in the conversion of urea to ammonia and that conversion of ammonia is not rate limiting, the theoretical energy available from 22 g of urea is:

$$\frac{22}{60}\ \text{mole} \times 6\ \frac{\text{equiv.}}{\text{mole}} \times 26.8\ \frac{\text{amp hr.}}{\text{equiv.}} \times 1.13\ V = 66.6\ \text{watt hr.}$$

A reasonable voltage efficiency for this cell appears to be 70%, so that energy from urea in urine that may be available as electrical energy is 47 watt hours per man-day. Power available would be approximately 2 watts per man.

Similar calculations for the minor organic components of urine must consider the electrochemically active intermediate and the electrode reaction that would be used. One approach is to assume a two-electron redox reaction for each of the minor components; the other is to assume that all the nitrogen could be converted to ammonia. Again, without regard to mechanism, estimates can be made of the energy of these compounds that might be converted to electrical energy.

The following tabulation gives estimates of the electrical energy and power made within the context of the foregoing assumption. A working cell voltage of 0.79 volt has been assumed for all cases. This value is 70% of the E° for the ammonia-oxygen reaction. It can be seen then, that urea is by far the most promising fuel component of urine.

Component	2-electron change		Conversion to ammonia	
	Power, Watts/man-day	Energy, Watt hrs/man-day	Power, Watts/man-day	Energy, Watt hr/man-day
Amino acids (taken as 1/2 alanine, 1/2 phenylalanine)	0.04	0.91	0.06	1.4
Creatinine	0.02	0.55	0.1	2.5
Hippuric acid	0.015	0.36	0.02	0.53
TOTALS	0.075	1.82	0.18	4.4

4.3.1.2 Feces

There is very little specific information on the composition of feces. Thus, an estimate of heat of combustion by summing heats of combustion of components is not possible. An estimate in the literature, however, is that the caloric content of feces is 70-140 Kcal per man-day; this corresponds to 81.5 to 163 watt hours per man-day.

An analysis of feces as an electrochemical fuel is considerably more difficult than for urine, primarily because information on the composition of feces is limited. By applying certain assumptions, it is possible to arrive at useful numbers, however.

The first consideration must again be given to what electrochemically active intermediates can be produced from feces, the components of feces themselves being inactive. There are a large variety of compounds present in feces in only small quantities. The significant components of feces are the nitrogen content, lipid content, and polysaccharide content. The nitrogen content of dry feces has been estimated at 6%. Lipids amount to 15-25% of dry feces and polysaccharides to 15-30%. The polysaccharide content is perhaps most subject to variation. These values for the most part represent not only discrete compounds and undigested residues, but also the live and dead bacterial matter present in feces.

Electrochemically active intermediates which might be produced from these major components of feces are largely ammonia and hydrogen. A limited basis exists for conversion of nitrogenous compounds to ammonia. Somewhat firmer grounds exist for hypothesizing hydrogen production from polysaccharides. No biological methods are known that could even remotely be considered to convert the lipids to electrochemically active intermediates to any significant degree. Because of the large number of compounds present, discrete enzyme-catalyzed reactions have been rejected for their specificity.

For the purposes of discussion, the complete conversion of nitrogen to ammonia can be assumed, although attainment of such complete conversion seems unlikely. Based on an average daily output of 150 g feces by an individual and an average solids content of 33%, average production of nitrogen is 3 g. Using assumptions described earlier for electrochemical utilization of ammonia, it is estimated that energy might be produced from feces at the rate of 14 watt hours per man-day.

On the basis that all the polysaccharide could be converted to glucose, and from the best data for hydrogen production from glucose (2.3 moles H_2 per mole glucose), the maximum polysaccharide content of feces is equivalent to 0.19 mole hydrogen per man-day. Using 0.8 volt as a working cell voltage for a hydrogen-oxygen cell, this quantity of hydrogen represents 8.2 watt hours per man-day.

4.4 Selection of Bacteria and Enzymes

Utilization of urine and feces as electrochemical fuels requires a mechanism for conversion of the electrochemically inactive components of these wastes to electrochemically active products, e.g., ammonia and hydrogen.

The simultaneous conversion of waste materials to reusable chemicals probably will require a different line of attack since optimization for both electrochemical fuels and waste disposal does not appear to be feasible. Optimum anaerobic digestion

processes yield methane, hydrogen sulfide, pyrimidines and other toxic reduced organic compounds which are not tolerable in a closed system. Appreciable quantities of ammonia and hydrogen are not produced. Aerobic processes, on the other hand, yield carbon dioxide, and only marginal amounts of ammonia.

For ammonia production, the urea bacteria in general were selected for screening. It was planned to narrow this selection in preliminary screening for growth in urine and accompanying ureolytic activity. The organisms surveyed include Sarcina ureae, Micrococcus ureae, Lactobacillus bifidus var. ureolyticus, Brevibacterium ammoniagenes, and Bacillus pasteurii. The prospect of converting fecal nitrogenous compounds to ammonia is considerably less attractive and was not pursued in this study.

For production of hydrogen from fecal saccharides, E. coli and Cl. perfringens were selected; both are indigenous to feces. The prospect of using mixed sewage cultures to produce hydrogen from feces held some promise and will be discussed in a later section.

As to enzymes, cellulase and lipase were selected for further study. Cellulase is of interest because (1) bacteria hydrolyze cellulose slowly and (2) the glucose from cellulose is a good source of hydrogen by way of bacterial fermentation. Lipase is of interest primarily because of the relatively high lipid content of feces. Because proteolytic activity is common in bacteria, papain was rejected for further consideration. Urease was rejected because urea bacteria exhibit high ureolytic activity. The pectic enzymes, pectinesterase and polygalacturonase, would be of limited value because of the small quantity of pectins in wastes.

Practical considerations lead also to the desirability of using urine and feces in mixtures. To this end, studies were concerned, on the one hand, with the effect of feces on bacterial production of ammonia from urine. On the other hand, admixture of urine with feces is indicated for work on production of hydrogen from fecal components, because feces, despite its high nitrogen content, is notoriously poor in nitrogen compounds that can readily contribute to bacterial metabolism.

4.5 Screening Program - Ureolytic Organisms

Screening bacteria for growth and attendant ureolysis in urine was performed to select the most favorable organism(s). Ureolysis was considered of greatest interest under anaerobic conditions, because electrochemical utilization of the produced ammonia would be expected to proceed most favorably in the absence of oxygen. Further, it is desirable to minimize oxygen utilization in a closed ecology.

Sarcina ureae - This organism grew and produced ammonia in aerobic but not in anaerobic cultures when Urea broth and urine were used as media. Consequently, S. ureae was eliminated from further study.

Micrococcus ureae - The culture, received from a commercial culture collection, was contaminated; however, since the literature indicated that M. ureae, like S. ureae, is essentially an aerobe, the organism was rejected on this basis.

Lactobacillus bifidus var. ureolyticus - Although the literature reports that this organism anaerobically hydrolyzes urea to ammonia (43), our culture failed to demonstrate ureolytic activity despite numerous subcultures in a medium containing urea. The investigator from whom the culture was obtained suspected that ureolytic ability was unstable and prolonged stock (5 years) of the culture probably contributed to the loss of this characteristic. No other source of this organism is known.

Bacillus pasteurii - This organism demonstrated aerobic and anaerobic ammonia production in Urea broth and urine. Somewhat better growth occurred aerobically in Urea broth while anaerobic growth was better in urine. After one day of anaerobic growth, approximately 97% of the urea present in urine was utilized.

Brevibacterium ammoniagenes - The bacterium grew readily and produced ammonia aerobically and anaerobically in both Urea broth and urine. Aerobic ureolytic activity was rapid (24-48 hours) in Urea broth and in urine, but ammonia was not evident before 5 days of anaerobic growth in urine. In the latter case, 46% of the urea present in the urine was utilized. The relatively slow ureolytic activity under anaerobic conditions resulted in our dropping this organism from further consideration.

The experimental screening program resulted in the selection of B. pasteurii for subsequent studies. This organism was selected for its relatively rapid anaerobic growth in urine accompanied by efficient urea hydrolysis.

4.6 Optimization of Ureolytic Activity

A series of experiments were carried out in batch culture, to establish optimum ureolytic parameters for growth of B. pasteurii in urine under anaerobic conditions for application to subsequent continuous culture and electrochemical studies.

4.6.1 Analytical Methods

An experiment was performed in which B. pasteurii was grown in urine in Warburg vessels to determine the quantity of free gas evolved. The over-all gas evolution was negligible, indicating that the carbon dioxide and ammonia produced from urea hydrolysis are effectively kept in solution as ammonium carbonate; this permits the use of an analytical technique for ammonium ion. The method selected was the colorimetric determination of Gentzkow and Masen (132). Warburg manometry may be useful, however, to measure dissolved carbon dioxide by terminal acidification of the cultures. The data is presented in Table 5.

The colorimetric analysis of Ormsby (133) was selected for the determination of urea in urine because of its relative simplicity and accuracy.

Since utilization of urea and production of ammonia were the metabolic reactions of greatest interest for subsequent electrochemical experiments, these were used as the prime indications of growth, except in some early experiments performed prior to development of these analytical techniques. In such cases, total cell number was measured to determine growth, either alone, or in a few cases combined with urea determinations.

TABLE 5

Gas Production Demonstrated Manometrically by Cultures of *B. pasteurii*

	Initial Values		Final Values (4 days)		
Flask No.	Urea (mg/ml)	pH	Urea (mg/ml)	pH	Approx. Gas Produced (ml of gas/10 ml of culture)
1	10.75	8.8	0.29	9.3	2.0
2	10.75	8.8	0.28	9.3	2.0
3	10.75	8.8	0.29	9.3	2.0

The general procedure for examining growth of B. pasteurii in static culture consisted of inoculating filter-sterilized (Seitz) urine with a 1% inoculum grown in urine. Cultures were usually grown in 30-ml screw cap tubes filled to capacity to exclude oxygen. Stock cultures were subcultured routinely in urine. Urine was analyzed for urea and ammonia both prior to inoculation and after growth which was generally 24 hours.

The incubation temperature was 30°C and the pH was usually adjusted to 8.8 - 9.0 after inoculation.

Using these techniques, the growth of B. pasteurii in urine has been shown to result in virtually 100% conversion of urea to ammonia. The mole ratio of ammonia produced to urea utilized is approximately 1.8:1 - 1.95:1 demonstrating that only a small amount of ammonia is converted to cellular nitrogenous material. Ureolysis is primarily an energy-yielding reaction.

4.6.2 Storage of Urine

It was established early in the program that there was no difference in anaerobic growth of B. pasteurii when either freshly collected, refrigerated (several days) or frozen urine was used as the medium (Table 6).

In order to maintain large quantities of urine under constant conditions for subsequent studies, urine collected from suitable donors was pooled and frozen. Prior to each experiment, a portion of the urine was rapidly thawed and sterilized by filtration.

4.6.3 pH

Bornside and Kallio (134) reported that the optimum pH for growth of B. pasteurii in a medium containing urea was pH 8.8. An experiment was performed which demonstrated that an initial pH of 9.0 supported optimal anaerobic growth in urine after 24 hours growth (Table 7). The pH of ammonium

TABLE 6

Comparative Growth of *B. pasteurii* in Fresh, Refrigerated, and Frozen Urine

	History of Urine		
	Fresh	Refrigerated	Frozen
Average final cell counts (cells/ml)	1.5×10^7	1.5×10^7	1.0×10^7

TABLE 7

Effect of pH on Growth[1] of *B. pasteurii* in Urine

Initial pH	7.5	8.0	8.5	9.0	9.5
Days after Inoculation					
1	7.3×10^5	3.8×10^6	2.7×10^6	1.4×10^7	3.8×10^6
2	1.9×10^7	3.2×10^7	2.4×10^7	2.3×10^7	2.4×10^7
3	1.3×10^7	1.2×10^7	2.1×10^7	1.4×10^7	1.6×10^7
Final pH	9.5	9.5	9.5	9.5	9.6

1 Average total cell counts on duplicate samples (cells/ml)

carbonate, produced from urea hydrolysis, is approximately 9.2 - 9.5 and solutions containing this material are strongly buffered in this pH range. At 48 and 72 hours, essentially no difference in growth was evident between cultures because the pH was identical.

4.6.4 Temperature

Temperature optimization studies with B. pasteurii grown in urine showed that urea utilization and ammonia production were inhibited at 25°C compared to growth at 30°C and 35°C (Table 8). No significant difference in total cell counts was observed between cultures grown at the higher temperatures; therefore, 30°C was selected as being more convenient for all studies.

4.6.5 Vitamins

Wiley and Stokes (42) showed that most strains of B. pasteurii require thiamine and certain amino acids for growth and that some strains also need biotin and nicotinic acid. Their cultures were grown in a synthetic medium to which vitamins were added selectively. Our studies (Table 9) established that growth was not enhanced by supplementing urine with these three vitamins, either alone or in combination. This would indicate that urine contains sufficient amounts of these vitamins as might be expected, since urine generally contains various amounts of all these vitamins.

4.6.6 Addition of Urea to Urine

An extensive study was made on the effect of adding extra urea to urine as a preliminary step in the use of concentrated urine. It was expected that this approach would enable work with smaller volumes of urine and also result in reduced electrical resistance of urine. Normal urine,

TABLE 8

Effect of Temperature on Growth of *B. pasteurii* in Urine

°C	Days After Inoculation	Ave. Urea[3] Utilized mg/ml	% Urea Utilized	Ave. NH_3 Produced[3] mg/ml	Ave. Final cell Count cells/ml	Mole Ratio Urea/NH_3
25[1]	1	4.70	38.4	1.49	2.2×10^7	1:0.114
25[1]	1	3.30	26.9	1.91	3.6×10^7	1:0.204
25[2]	2	10.59	86.4	5.57	-	1:1.86
25[2]	2	10.15	84.5	5.04	-	1:1.76
30[1]	1	11.66	95.4	6.16	4.2×10^7	1:1.88
30[1]	1	11.72	95.6	6.16	6.2×10^7	1:1.85
30[2]	2	12.01	98.0	5.83	-	1:1.72
30[2]	2	12.01	98.0	6.19	-	1:1.82
35[1]	1	11.64	95.2	6.10	3.6×10^7	1:1.85
35[1]	1	11.72	95.6	6.20	5.2×10^7	1:1.86
35[2]	2	12.01	98.0	6.02	-	1:1.77

1 Experiment 1

2 Experiment 2

3 Initial urea - 12.25 mg/ml

TABLE 9

The Effect of Added Vitamins on Growth[1] of *B. pasteurii* in Urine

Vitamin Added	Normal Urine (control)	Nicotinic Acid	Thiamine hydrochloride	Biotin	Nicotinic Acid & Biotin	Biotin & Thiamine hydrochloride	Nicotinic Acid & Thiamine hydrochloride	Nicotinic Acid, Thiamine hydrochloride & Biotin
Days after Inoculation								
1	1.1×10^7	2.1×10^7	1.6×10^7	1.8×10^7	1.6×10^7	2.2×10^7	1.4×10^7	1.7×10^7
2	2.4×10^7	1.3×10^7	1.9×10^7	2.0×10^7	1.7×10^7	1.6×10^7	2.0×10^7	1.6×10^7
Final pH	9.5	9.5	9.5	9.5	9.5	9.5	9.5	9.5

1 Average of total cell counts from duplicate tubes (cells/ml)

containing 1.0% urea was supplemented at various levels up to 3.9% urea. Table 10 shows that urea utilization and urea concentration varied inversely. Urea utilization decreased from 96% to 58% over the range of urea concentration examined. At urea concentrations higher than 2.7%, urea utilization did not improve, while ammonia production did not increase at urea concentrations above 1.9%. Up to 1.9% urea, the mole ratio of ammonia produced to urea utilized was approximately 1.9:1; this ratio decreased to 1.7:1 at the highest urea concentration used. The total number of cells was approximately the same at all urea levels which emphasizes the need for urea and ammonia analyses for a more accurate representation of cultural performance.

Based on the amount of ammonia produced, the data indicate that 1.9% urea appears to be the optimum concentration, since no improvement occurs at higher concentrations of urea. It is expected that by employing adaption procedures, B. pasteurii could be "trained" to tolerate higher levels of urea.

4.6.7 Addition of Feces to Urine

Although B. pasteurii grows fairly well in urine alone, it is likely that supplementing urine with those nutrients that are growth-limiting will improve growth and probably the rate of ureolysis. Urine is composed largely of inorganic compounds and contains only marginal quantities of organic carbonaceous material from which heterotrophic bacteria such as B. pasteurii must synthesize the bulk of their cell protoplasm. Urea supplies an energy and nitrogen source, but not a source of carbon. A possible approach to providing utilizable, organic material is the addition of feces to urine.

Experiment 1

The study was initiated by growing B. pasteurii in urine with 4, 20 and 50% whole feces added. The highest urea

TABLE 10

Effect of Urea Concentration on Growth of B. pasteurii in Urine

% Urea	Ave. Urea Utilized mg/ml	% Urea Utilized	Ave. NH_3 Produced[3] mg/ml	Ave. Final Cell Count	Mole Ratio Urea/NH_3
1.0[2,3]	9.87	96.5	5.24	3.0×10^7	1:1.87
1.0[2]	9.85	96.2	5.08	2.5×10^7	1:1.83
1.4[1]	13.48	9 3.8	7.22	5.9×10^7	1:1.89
1.4[1]	13.40	93.4	7.11	5.8×10^7	1:1.88
1.9[2]	17.43	89.0	9.74	2.8×10^7	1:1.69
1.9[2]	18.53	94.5	9.92	2.4×10^7	1:1.85
2.7[2]	21.06	78.8	9.92	3.5×10^7	1:1.66
2.7[2]	21.31	79.7	10.76	2.4×10^7	1:1.79
3.1[1]	20.93	68.6	9.63	4.6×10^7	1:1.62
3.1[1]	21.68	69.0	10.43	5.7×10^7	1:1.70
3.5[2]	22.37	64.6	10.65	2.4×10^7	1:1.68
3.5[2]	21.00	60.9	9.74	1.5×10^7	1:1.70
3.9[2]	22.65	57.7	10.63	1.8×10^7	1:1.71
3.9[2]	22.75	58.0	11.15	2.3×10^7	1:1.73

1 Experiment I

2 Experiment 2

3 Normal urine

utilization occurred in the culture with 4% feces, approximately 95% of the urea being utilized (Table II). In the 20% and 50% feces cultures, 89 and 80% of the urea, respectively, was consumed. In urine alone, 98% of the urea was consumed. Although not measured quantitatively, the odor of ammonia was detected in all cultures.

Experiment 2

A second experiment was performed to provide data on the amounts of ammonia produced and urea utilized. B. pasteurii was cultured in urine containing 1, 5 and 10% lyophilized feces. Approximately the same amount of ammonia was produced in the 1 and 5% feces cultures as in the control (urine alone) culture (Table 12). Somewhat reduced ammonia production occurred in the 10% feces culture. An elevation of the urea determinations for this and the previous experiment indicated that feces interfered with the analysis; therefore, the urea data must be considered only approximate. No such interference was evident for the ammonia analysis. The results indicate that the addition of feces to urine do not enhance ammonia production; in fact, at the highest concentration examined, inhibition was observed.

Experiment 3

The inability of feces to enhance growth of B. pasteurii in urine might be attributed to toxic compounds present in feces. In an attempt to overcome toxicity and/or encourage expression of adaptive enzymes, B. pasteurii

TABLE II

Effect of Feces on Growth of *B. pasteurii* in Urine

Experiment 1

Whole Feces by Weight (%)	Ave. Initial Urea mg/ml	Ave. Urea Utilized mg/ml	% Urea Utilized	Final pH
4	8.0	7.59	95.0	9.2
4	8.0	7.61	95.2	9.2
20	7.5	6.52	88.0	9.1
20	7.5	6.70	89.5	9.1
50	7.0	5.62	80.3	8.8
50	7.0	5.62	80.3	9.0

TABLE 12

Effect of Feces on Growth of B. pasteurii in Urine

Experiment 2

Lyophilized Feces by Weight (%)	Ave. Initial Urea mg/ml	Ave. Urea Utilized mg/ml	% Urea Utilized	Ave. NH_3 Produced mg/ml	Mole Ratio Urea/NH_3
0	12.75	12.41	97.5	5.44	1:1.54
0	12.75	12.44	97.6	5.75	1:1.64
1	15.00	14.42	96.4	5.92	1:1.51
1	15.00	14.45	96.4	5.46	1:1.33
5	12.00	10.30	85.5	5.34	1:1.83
5	12.00	10.35	86.4	5.64	1:1.91
10	10.25	7.70	75.0	4.92	1:2.26
10	10.25	8.05	58.5	5.12	1:2.24

was subcultured 5 times in a medium consisting of 5% lyophilized feces in urine. The previous experiment was then repeated using this B. pasteurii culture as the inoculum. The data, however, was very similar to earlier results (Table 13). No enhancement of ammonia production was observed and inhibition occurred at the highest concentration of feces in urine (10%).

It appears that unaltered feces does not contribute beneficially to the nutrition of B. pasteurii growing in urine. Chemical or physical pretreatment may possibly convert feces to a more suitable substrate but this remains to be investigated.

4.6.8 Continuous Culture of B. pasteurii

The next major phase of the study was the growth of B. pasteurii in continuous culture. To fully appreciate the value of continuous culture in the study of microbial physiology, this technique must be compared with batch or stationary culture techniques.

A normal microbial batch culture is a closed system. After an initial period of adjustment, growth becomes steady and rapid for a period which is referred to as the exponential growth phase. During this phase all the components of the microbial cell are, in mathematical terms, increased by the same factor. This steady state of growth is temporary, however, and ceases when nutrient concentration drops to limiting values or when metabolic products reach toxic concentrations.

Practical means for the maintenance of steady state for periods of long duration have been devised and numerous successful methods have been described (135). Essentially they provide conditions for the continual supply

TABLE 13

Effect of Feces on Growth of *B. pasteurii* in Urine

Experiment 3

Lyophilized Feces by Weight (%)	Initial Urea mg/ml	Ave. Urea Utilized mg/ml	% Urea Utilized	Ave. NH_3 Produced mg/ml	Mole Ratio Urea/NH_3
0	11.88	11.51	97.0	5.75	1:1.76
0	11.88	11.48	97.2	5.39	1:1.65
1	10.75	10.31	96.0	5.35	1:1.82
1	10.75	10.35	96.5	5.20	1:1.77
5	12.25	11.65	95.2	5.19	1:1.57
5	12.25	11.62	95.0	5.54	1:1.68
10	10.88	8.63	79.5	5.25	1:2.14
10	10.88	8.60	79.0	5.20	1:2.14

of fresh medium and the simultaneous removal of an equal volume of growing culture maintained at constant volume. The value of this technique is that microbial growth now occurs under steady state conditions for prolonged periods, i.e., growth takes place at a constant rate and in a stable, reproducible environment. Such variable factors as nutrient concentration, oxygen, pH, and metabolic products, which eventually change during the growth cycle in batch culture, are all maintained constant in continuous culture.

Early methods of extending the exponential phase of growth depended upon growing microorganisms within dialysis bags or adding some adsorbent to a culture for the adsorption of toxic metabolic products.

Further exploration eventually led to two divergent methods for the control of continuous culture. One approach, exemplified by the Turbidostat (136), is referred to as internal control and attains the steady state through regulation of nutrient feed controlled by optically sensing turbidity of the culture. Thus, the dilution rate varies with the population density of the culture and fluctuates about a mean, maintaining the density within a narrow range. It is obvious that this method cannot be used when growth produces a precipitate as is the case of B. pasteurii cultured in urine. The second means for obtaining steady state growth utilizes the principle that growth of an organism is limited by nutrient concentration below certain values. A constant feed of medium with one nutrient in limiting concentration with constant removal of culture at the same rate is used to achieve steady state. This continuous flow system permits the selection of a desired population density by regulation of a limiting concentration of nutrient flowing into the microbial culture and the selection of a desired growth rate by regulation of the flow rate. This method is referred to as external control; the Chemostat of Novick (137) and the Bactogen of Monad (138) are two examples of this type of continuous culture apparatus.

In the chemostat the dilution rate is set at an arbitrary value and the microbial population allowed to find its own level; by appropriate setting

of the dilution rate, the growth rate may be held at any desired value from slightly below the maximum possible to nearly zero.

The cell population grows exponentially but the steady state is maintained constant at a value somewhat less than the maximum growth rate by virtue of employing a limiting concentration of nutrient. This, in effect, constitutes a self-regulating system and allows selection of a desired growth rate. A wide variety of substances can serve to limit or control growth. The nutrients most commonly employed for limitation are (a) amino acids, (b) energy sources, (c) nitrogen sources, (d) phosphate ion, and (e) sulfate ion. The virtue of the chemostat is that growth can be studied at very low concentrations of nutrient and over a wide range of growth rates. It is known that the morphological and physiological properties of cells change simultaneously with their growth rate. For example, it has been shown that Azotobacter vinelandii metabolized glucose and utilized oxygen more efficiently with increasing growth rates (139). Other studies have demonstrated that RNA content of bacteria increases with growth rate while DNA content decreases with increasing growth rate. In addition, fast-growing cells are larger and have a greater mean cell mass than slow-growing cells. These studies serve to illustrate the relationship between growth rate and other properties of microorganisms such as chemical composition, dimensions and physiological-biochemical properties.

Because externally controlled systems (chemostat) allow variation in the growth rate, growth rate in itself becomes an important experimental parameter. For example, it is possible to establish a quantitative as well as a qualitative relationship between the composition of the culture medium and growth rate by selecting the optimum composition for growth. This may be accomplished by varying the concentration of individual components of the medium and determining the effects on growth rate of the culture. In the chemostat low concentrations of chemicals can be maintained constant even though they might be utilized at high rates. The same general method can be adapted to rapidly determine the effect of bacteriostatic agents and anti-metabolites on growth rate.

Another application of the chemostat which takes advantage of the fact that low concentrations of a chemical can be maintained constant over many generations, is the study of the kinetics of induction of an inducible enzyme. The concentration of inducer is maintained at a desired value and the microorganisms are removed periodically and assayed for enzyme activity. By this means it is possible to change the metabolic activity of the cell population to the advantage of the particular process under study.

In conclusion, it can be seen that unlike conventional batch culture, continuous culture by virtue of creating a constant environment permits the experimenter to establish rapid and precise relationships between the environment and the activities of the microbial population. These include the morphology, chemical composition, growth rate, and physiological-biochemical activities of microorganisms.

Since one of the major objectives of the ensuing continuous culture studies was to establish base-line data with normal urine, the urine was not modified or supplemented in any manner. The continuous cultures were chemostatically controlled by means of some undefined component of the urine acting as the growth-limiting nutrient. The limiting component was most likely an organic carbon source, since urine contains mostly inorganic ions.

Experiment I

A preliminary experiment was performed to learn major operational parameters and to determine whether modifications in the design of the apparatus would be necessary. Urea utilization and production of ammonia were used to gauge performance of the culture. The culture was sampled intermittantly from the overflow tube. During the 5 day run, it was observed that decreasing the flow rate from a maximum of 19 ml per hour to 13 ml per hour resulted in increased efficiency in conversion of urea to ammonia (Table 14). Ammonia production almost doubled during this

TABLE 14

Growth of B. pasteurii in Continuous Culture

Experiment 1

Age of Culture (hrs)	Flow Rate ml/hr.	Ave. NH_3 Produced[2] mg/ml	Ave. Urea Utilized[1] mg/ml	% Urea Utilized	Mole Ratio Urea/NH_3
5		2.65	4.62	38.4	1:1.99
22	19.4	2.96	4.93	41.0	1:2.1
29		3.23	4.50	37.5	1:2.53
46	17	4.52	7.50	62.5	1:2.12
51		4.26	8.00	66.5	1:1.88
121	13	5.34	10.8	87.0	1:1.74

1 Initial urea of inoculum, 5.25 mg/ml

2 Initial NH_3 of inoculum, 2.77 mg/ml

period and urea utilization increased from 49% to 87%. The mole ratio of urea utilized to ammonia produced stabilized at approximately 1:2 during the 5 day run. The greatest difficulty observed during this experiment was the formation of a heavy precipitate which deposited on the walls of the growth chamber and plugged the orifice of the overflow tube.

Experiment 2

To overcome the problem of precipitate plugging the orifice, the bore of the overflow tube was enlarged and a magnetically-controlled polyurethane pad was placed inside the vessel so that the precipitate could be scrubbed from the walls when necessary.

The continuous culture was operated for 19 days. Table 15 shows that maximum utilization of urea was 89.5%, which occurred on the 19th day of operation, at a retention time of 10 hours (flow rate of 9.6 ml per hour). As in the previous experiment, the mole ratio of urea utilized to ammonia produced stabilized at approximately 1:2 during the latter portion of the run.

Two new problems were encountered during this experiment. Urea utilization was greater in samples collected from the overflow than in samples taken directly from the growth vessel which demonstrated that a discontinuity existed between cultural conditions in these two areas. Therefore, subsequent sampling was made directly from the growth vessel.

TABLE 15

Growth of B. pasteurii in Continuous Culture

Experiment 2

Age of Culture (days)	Flow Rate ml/hr	Ave. NH_3 Produced[2] mg/ml	Ave. Urea Utilized[1] mg/ml	% Urea Utilized	Mole Ratio Urea/NH_3
3	12.3	4.67	8.78	69.5	1:1.88
4	11.5	4.90	9.43	75.0	1:1.75
5	6.9	4.27	10.45	76.0	1:1.44
6	17.0	3.13	7.85	67.0	1:1.42
7	17.0	4.00	8.35	60.6	1:1.69
10 (overflow)	13.2	5.22	9.65	75.4	1:1.91
10 (growth vessel	13.2	4.44	8.23	65.0	1:1.90
11	13.1	4.52	9.20	72.7	1:1.82
12	12.1	6.19	9.85	77.8	1:2.2
13	12.3	5.59	10.43	82.5	1:1.89
14	13.3	5.51	10.60	85.0	1:1.83
19 (overflow)	9.6	5.35	10.95	89.5	1:1.72
19 (growth vessel)	9.6	5.20	9.80	79.3	1:1.89

1 Initial urea of inoculum, 6.69 mg/ml

2 Initial NH_3 of inoculum, 3.17 mg/ml

The flow rate of urine varied considerably due to the inability of the Sigma pump to feed urine at a constant rate. Consequently, the continuous culture did not reach a steady state for any significant length of time. The Sigma pump was replaced with a Harvard Infusion-Withdrawal pump designed to deliver low rates of liquid with a high degree of accuracy.

Experiment 3

The next continuous culture was operated for 17 days. As the flow rate decreased, the efficiency of urea utilization increased until a steady state was maintained for a 4 day period between the 11th and 15th day of operation (Table 16). 83-84% of the urea was utilized at a retention time of approximately 12 hours (flow rate of 8.3 ml per hour). The ammonia produced varied from 0.45 to 0.49 mg per ml of culture per hour. The stoichiometry of urea utilized to ammonia produced was slightly over 1:2, except on the 15th day, when the ratio jumped unexplainedly to 1:2.39.

The experiment was terminated when mold contamination was observed in the feed tubing.

Experiment 4

The final continuous culture in this series was operated for 15 days, 7 of which were at steady state, (Table 17). During this period, approximately 80% of the urea was utilized at a retention time which averaged 13.3 hours

TABLE 16

Growth of B. pasteurii in Continuous Culture

Experiment 3

Age of Culture (days)	Flow Rate ml/hr	Ave. NH_3 Produced[1] mg/ml	Ave. Urea Utilized[2] mg/ml	% Urea Utilized	Mole Ratio Urea/NH_3
3	11.5	4.47	6.68	62.6	1:2.36
4	11.5	4.58	7.20	67.6	1:2.24
7	10.6	5.13	8.65	81.2	1:2.09
8	10.0	5.85	8.67	81.5	1:2.38
9	10.0	4.97	7.63	71.5	1:2.28
10	8.3	5.12	8.53	80.0	1:2.12
11	8.0	5.52	8.93	84.0	1:2.18
14	8.4	5.40	8.80	84.0	1:2.16
15	8.5	5.88	8.72	83.0	1:2.39
16	7.5	5.60	9.10	86.5	1:2.16
17	5.5	5.53	8.54	82.7	1:2.07

1 Initial urea of inoculum, 5.55 mg/ml

2 Initial NH_3 of inoculum, 3.56 mg/ml

TABLE 17

Growth of B. pasteurii in Continuous Culture

Experiment 4

Age of Culture (days)	Flow Rate ml/hr	Ave. NH_3 Produced[1] mg/ml	Ave. Urea Utilized[2] mg/ml	% Urea Utilized	Mole Ratio Urea/NH_3
1	6.00	4.38	6.40	69.8	1:2.41
4	6.25	4.65	6.49	70.8	1:2.53
5	7.20	4.91	8.60	75.8	1:2.02
6	7.80	5.22	8.98	78.4	1:2.18
7	7.25	5.60	9.08	80.0	1:2.18
8	7.15	5.38	9.30	80.9	1:2.04
12	7.80	4.76	9.40	80.0	1:1.78
13	7.25	5.32	8.60	78.2	1:2.18

1 Initial urea of inoculum, 0.613 mg/ml

2 Initial NH_3 of inoculum, 5.95 mg/ml

(flow rate of 7.5 ml per hour). The average concentration of ammonia produced was approximately 0.41 mg per ml of culture per hour. As in the previous run, the mole ratio of urea utilized to ammonia produced was slightly over 1:2.

To summarize the results of the four continuous culture experiments, steady state was maintained up to 7 days at retention times which ranged from 12 to 13.3 hours. Efficiency of urea utilization ranged from 80 to 84% and ammonia produced ranged from 0.41 to 0.49 mg per ml of culture per hour. The solution was buffered at a pH of 9.2 as a result of the formation of ammonia carbonate.

It is not surprising that values approaching 100% efficiency of urea utilization were not attained because, as discussed earlier, chemostatically controlled continuous cultures do not achieve maximum rates of growth. Their rates of growth are controlled by controlling the level of a growth-limiting nutrient. Complete conversion of urea, however, may be accomplished by recycling the effluent back to the culture or by operating a multi-stage continuous culture. The multi-stage system involves a chain of cultures in which the effluent of each feeds the next; culture medium is fed to the first stage only.

4.6.9 Addition of EDTA to Urine

The formation of the heavy precipitate in urine caused considerable difficulty in the operation of continuous culture as discussed earlier. IR spectrophotometric analysis showed that the insoluble salts were a mixture of calcium phosphate, and calcium and/or magnesium carbonate.

Several experiments were performed in stationary culture to determine whether the addition of EDTA to urine would chelate calcium and magnesium ions and thereby prevent or reduce the formation of insoluble salts at alkaline pH values

Experiment 1

The first experiment was concerned with establishing the effective concentration of EDTA. As a preliminary to the growth study, it was found that the addition of a minimum of 0.15% EDTA was required to prevent precipitation in sterile urine when the pH was increased to 9.2 with NaOH. A subsequent growth study (Table 18) showed that the addition of this concentration of EDTA reduced but did not entirely prevent precipitation. EDTA was responsible for an extended lag in growth (4 days), but the final rate of ammonia production was not significantly different than in the control cultures lacking EDTA.

Experiment 2

In the next experiment, the concentration of EDTA was increased to 0.2%, since 0.15% was only partially effective. The concentration of the B. pasteurii inoculum was examined at 1%, 5%, and 10%, to determine whether a larger inoculum would overcome the lag period and consequently result in more rapid growth. Table 19 shows that although no precipitation occurred, a 4-day lag period resulted even with the 10% inoculum. Further, a 5% inoculum was the minimum concentration required to produce ammonia in amounts that compared favorably to the control cultures lacking EDTA.

In view of the toxicity exhibited by EDTA and the high concentrations required to prevent precipitation, it does not appear that this approach is practical. Even if the tocivity could be overcome by adaption techniques or other means, the large weight

TABLE 18

Growth of *B. pasteurii* in Urine Supplemented with EDTA

Experiment 1

Tube No.		Ave. NH_3 Produced mg/ml	Turbidity	Final pH
1.	Control (normal urine)	5.52	+4	9.4
2.	Control (normal urine)	6.14	+4	9.4
3.	0.1 g EDTA/100 ml	5.72	+3	9.4
4.	0.1 g EDTA/100 ml	5.92	+3	9.4
5.	0.15 g EDTA/100 ml	5.76	+2	9.4
6.	0.15 g EDTA/100 ml	5.86	+2	9.4

TABLE 19

Growth of *B. pasteurii* In Urine Suppmented with EDTA

Experiment 2

Tube No.	Ave. NH_3 Produced mg/ml		% Inoculum
	Control (normal urine)	EDTA (2.0 m/l)	
1	6.02	1.85	1
2	6.45	1.89	1
3	5.82	5.30	5
4	5.87	5.40	5
5	5.44	5.50	10
6	5.44	5.30	10

penalty imposed by the requirement for 2g of EDTA per liter of urine would be prohibitive in a closed ecological system.

4.7 Feces Studies

4.7.1 Production of Hydrogen from Feces

There are two divergent microbiological approaches that may be considered in an attempt to produce hydrogen from feces. The first employs a pure culture of a microbial species, such as E. coli, known to metabolize certain carbohydrates to yield hydrogen.

The second approach consists of using enrichment procedures with a mixed sewage culture and/or indigenous fecal microflora. The object, in this case, is to provide a selective environment which favors the development and ultimate dominance of hydrogen-producing organisms.

Hydrolytic enzymes such as cellulase and lipase may contribute to bacterial action by degrading complex organic materials to simpler compounds, particularly if the use of pure cultures is planned. The enzymes would be employed to pretreat feces for subsequent bacterial metabolism rather than used simultaneously with bacteria. An evaluation of the organic substrates found in feces suggests that the use of enrichment techniques may be a more promising first approach than pure cultures because feces contains virtually no low molecular weight carbohydrates. A mixed culture offers the advantage of nutritive beneficial associations in which the more complex carbohydrate polymers common to feces are degraded in a step-wise fashion to simpler, more nutritionally available compounds for hydrogen-producing organisms.

Experiment I

An attempt was made to produce hydrogen from feces by employing a sewage culture as the inoculum and providing conditions

favorable to hydrogen-producing bacteria. An inoculum consisting of treated sewage from a primary sewage plant was inoculated into a medium containing sterile lyophilized feces and urine. Urine was used in the medium to provide a nitrogen source (largely urea) since feces are poor in assimilable nitrogen. To encourage enrichment of hydrogen-producing organisms and inhibit the methane bacteria which normally dominate aerobic sewage fermentations, the pH of the culture was buffered at 5.5. The cultures were grown under anaerobic conditions in 3-liter macrorespirometers to measure gas formation. Controls consisting of the sewage inoculum without added feces or urine were required to distinguish between fermentation of fecal organic material and residual organic material present in the inoculum.

The fermentation of feces was partly successful in that moderate digestion of solids and considerable gas formation occurred, demonstrating that the inoculum was active (Table 20). However, although no methane was evolved, only trace amounts of hydrogen were detected, suggesting that either feces was deficient as a substrate for hydrogen evolution or that the inoculum was not suitable for this purpose.

Experiment 2

Another experiment was performed in a similar manner except that a feces-adapted anaerobic mixed culture was substituted for the primary sewage culture. An innovation in this study consisted of adding glucose to one set of cultures to determine whether hydrogen-producing organisms were present in the inoculum. Table 21 shows that significant hydrogen production occurred only in the glucose-containing cultures as contrasted to trace amounts of hydrogen produced in the cultures lacking glucose. This demonstrates that

TABLE 20

Anaerobic Fermentation of Feces-Urine Medium[1]

Experiment I

Digestor No.	1	2	3	4
(ml gas evolution at STP, days)	feces/urine	feces/urine	Control (no feces/urine)	Control (no feces/urine)
1	83.0	90.5	0	0
4	291.0	257.0	0	0
5	291.0	355.0	0	0
6	326.0	374.0	0	0
Final pH	5.9	5.8	6.2	6.2
Total solids g/l Initial	49.07	49.07	4.01	4.01
Final	34.46	32.99	3.98	4.06
% Reduction	29.90	32.70	0.75	1.20
Volatile solids g/l Initial	42.20	42.20	2.72	2.12
Final	26.87	24.51	1.54	1.56
% Reduction	36.40	42.00	27.40	26.40

1 Primary sewage used as inoculum

TABLE 21

Anaerobic Fermentation of Feces-Urine Medium
Experiments 2 and 3

Digestor No.	Glucose Added	Gas Produced (ml)	Hydrogen Produced	CH_4 Produced	Final pH
Feces-adapted anaerobic inoculum (Exper. 2)					
1	yes	96	yes	no	6.1
2	yes	220	yes	no	6.1
3	no	6	trace	yes	6.0
E. coli inoculum (Exper. 3)					
1	yes	6[1]	yes		7.4
2	yes	298	yes		6.5
3	no	-[2]	no		4.3
4	no	-	no		7.1

1 Probable blockage in tube connecting flask with manometer

2 Accidental contamination of culture with acidified water

although hydrogen-producing organisms were present, unaltered feces did not serve as a suitable substrate under the cultural conditions used in this experiment. Since the hydrogen yield was not quantitated, there remains the possibility that glucose encouraged the development of a microflora that fermented fecal polysaccharides to hydrogen, as well.

Buffering at acid values was adequate to suppress methane formation in the cultures containing glucose but a small amount of methane was detected in the culture lacking glucose.

Experiment 3

To complete this series of studies, the previous experiment was repeated, using a pure culture of a known hydrogen-producer, E. coli, as the inoculum. Again, significant amounts of hydrogen were produced only in cultures supplemented with glucose (Table 21).

These three experiments illustrate the need for biological or chemical pretreatment of feces to break down complex organic polymers to simple, fermentable substrates. For example, cellulose may be hydrolyzed to glucose by the enzyme cellulase.

4.7.2 Enzymatic Hydrolysis of Fecal Components

Both cellulase and lipase were examined as potentially useful for pretreatment of feces. The objective was to facilitate action of selected bacteria on feces through breakdown of complex fecal components. The enzyme program was considerably abreviated to comply with the December, 1963 decision (see Conferences), to terminate studies on feces as a fuel.

4.7.2.1 Cellulase

The initial step in the study was to develop a colorimetric assay for cellulase and obtain a satisfactory calibration curve. The assay developed by Sumner (140) is based on the reduction of 3,5-dinitrosalicylic acid by the "reducing sugars" released during the hydrolysis of cellulose. The resultant reduction product imparts a characteristic brown color to the solution.

Evaluation of commercially available cellulase showed that reducing sugars present in the preparation interfered with the colorimetric assay procedure for cellulase activity. The assay is dependent on production of reducing sugars from cellulose. Dialysis of the crude enzyme allowed removal of impurities to the extent that they could no longer be detected.

The influence of temperature on cellulase activity was studied at 30, 40, and 50°C. Representative data are shown in Figure 1. Over 3 hours of a 5-hour observation period, essentially no differences could be observed at the three temperatures; all data were within approximately 5%. During the last 2 hours, the 30°C run lagged the 40 and 50°C runs by approximately 10%. A sharp decrease in activity was seen during the first hour's observation. This break is believed due to accumulation of products such as cellobiose and glucose. Such inhibition can be overcome by retaining the reactive system (enzyme with cellulosic material) in a dialysis membrane through which low molecular weight products could diffuse readily.

Use of cellulase with feces is complicated by the brown color of feces interfering with the assay procedure. Attempts to decolorize feces with charcoal to facilitate assay were unsuccessful.

4.7.2.2 Lipase

Examination of a crude pancreatic lipase preparation showed good activity in an assay method using triacetin (glyceryl triacetate). Tests performed

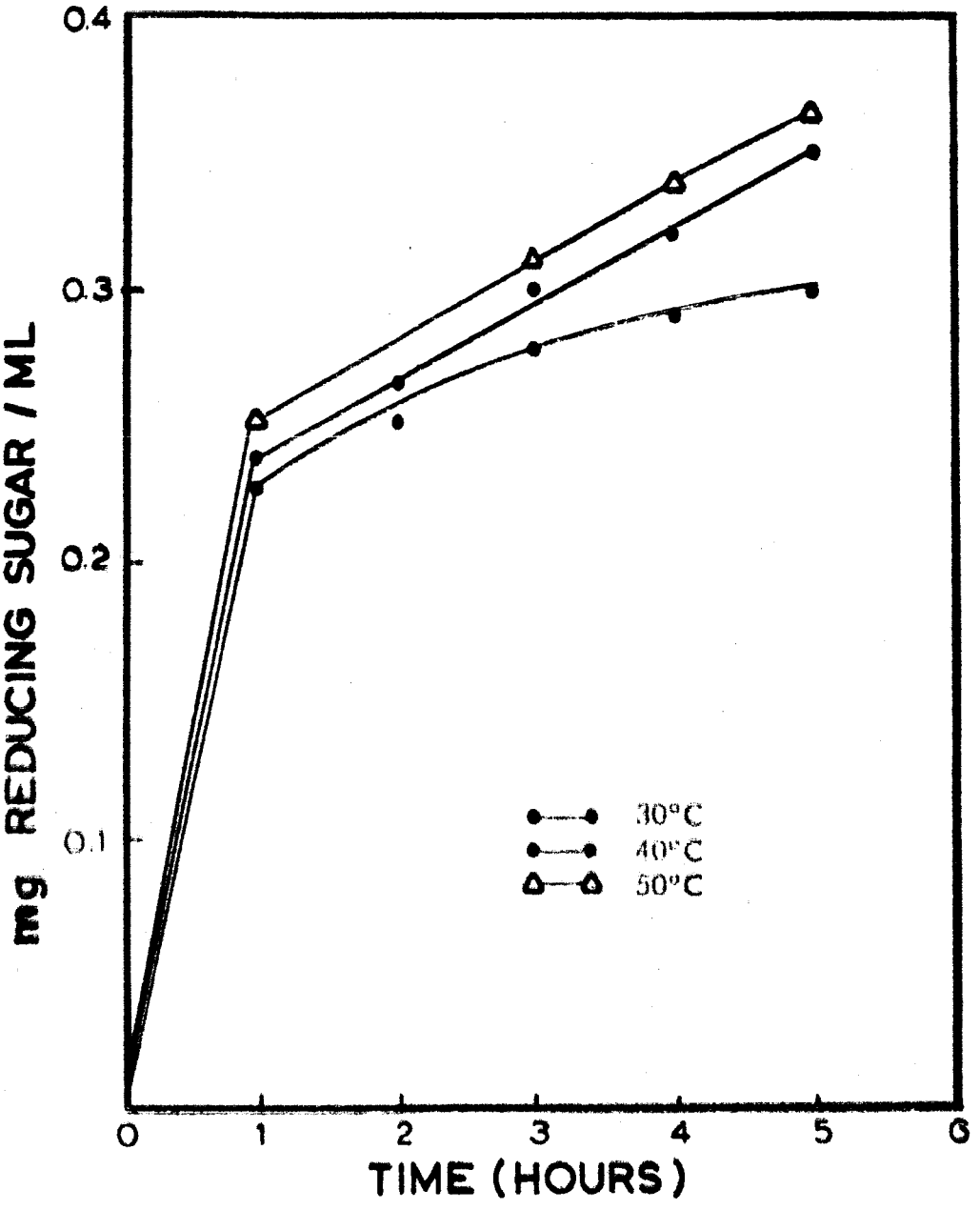


Figure 1

The Effect of Temperature on Cellulase Activity

with the enzyme and a mixture of 20% feces in urine resulted in activity comparable to that with triacetin. This is contrary to literature reports which suggest that feces inhibits lipase (13). Data for lipase activity both with triacetin and with the urine-feces mixture are shown in Figure 2.

4.8 Electrochemical Evaluation of B. pasteurii in Urine

The feasibility of electrochemical energy conversion systems is governed by the thermodynamics of the reactants. The actual performance of a system depends on the electrode kinetics of the cell. For example, the actual steady potential of an electrode usually differs markedly from the potential calculated from thermodynamic considerations; the cell current is the algaebraic sum of the oxidation and reduction currents at each electrode. Since each current is a measure of the rate of a particular reaction, the cell current is the resultant of the behavior of perhaps several oxidation and reduction reactions, under the influence of the potential across the electrodes, and the ensuing polarization effects.

Some cells, which are seemingly simple, exhibit complex performance. The obviously complex systems involved in bioelectrochemistry may show the effects of several simultaneous oxidation-reduction reactions in addition to the ramifications from the various types of polarization.

4.8.1 Half-Cell Studies

In the first section of this study the electrochemical behavior of systems composed of urine and B. pasteurii were observed. Urine was used directly rather than working with simpler systems employing urea because pure urea is being carefully examined under Contract DA 36-039 SC-90866. This study will be extended, and the conclusions derived will form the basis for the projected optimization of bioelectrochemical cells utilizing urine and feces.

The first data obtained, shown in Figure 3, were for systems containing non-sterile urine with and without B. pasteurii. There was little difference

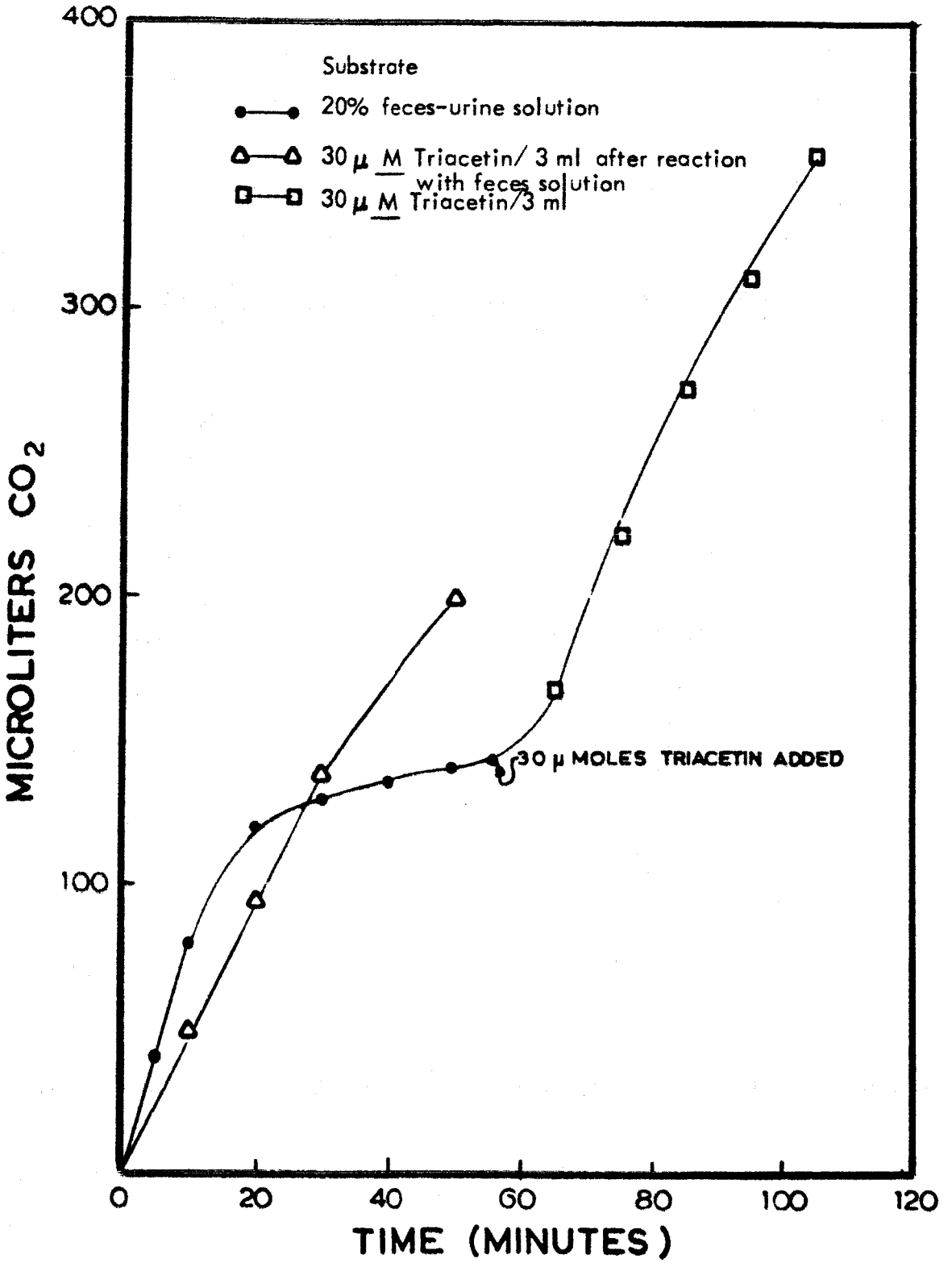


Figure 2

Lipase Activity with Triacetin and Urine-Feces Mixture

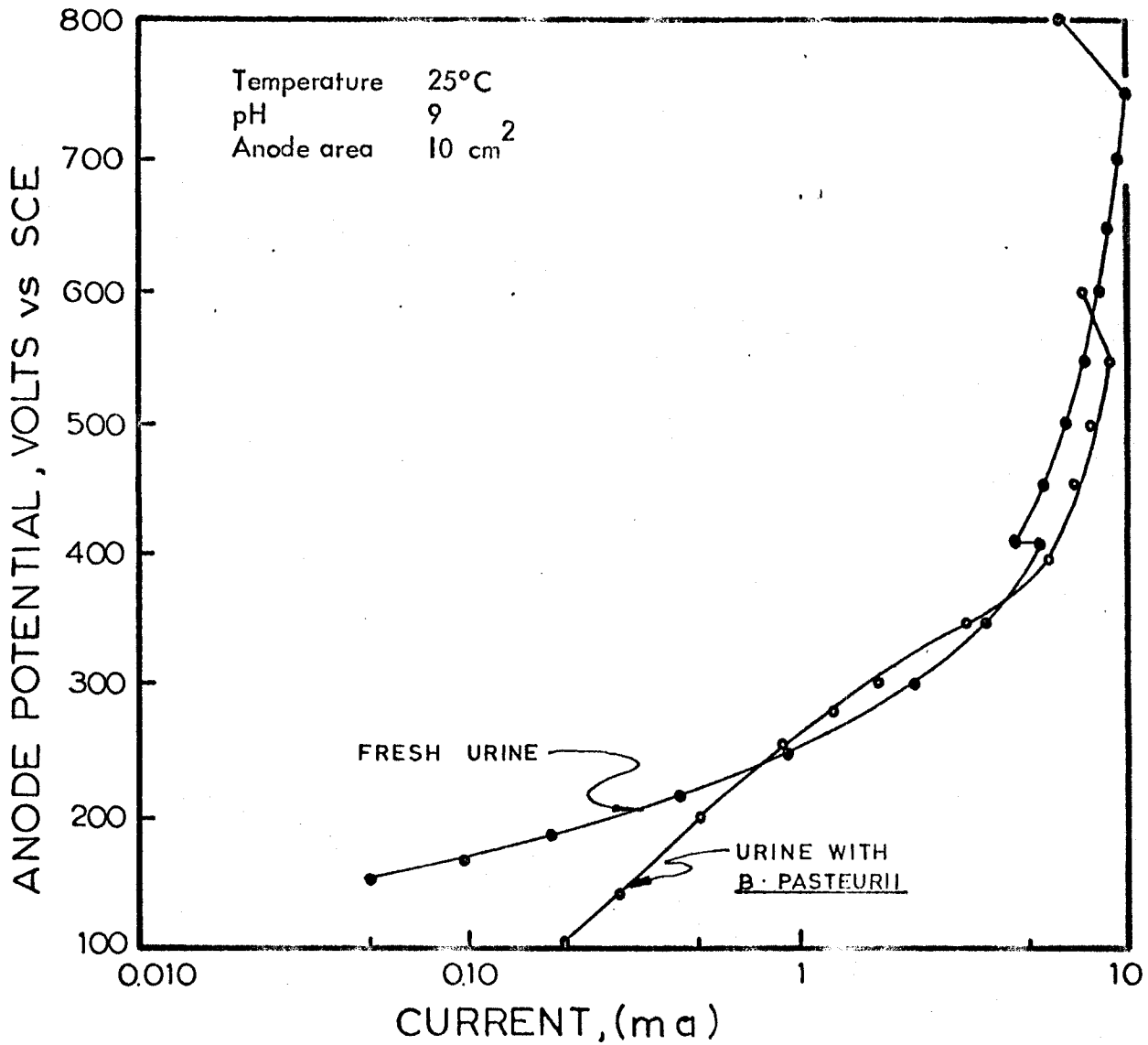


FIGURE 3: Anodic Polarization of Platinum in Urine, Effect of Added B. pasteurii

between the anodic polarization curves for these two sets of data at potentials more positive than 250 mV. The systems behaved nearly identically and the limiting current densities were the same. At potentials more negative than 250 mV, the curves are linear and have different slopes. The non-equal slopes in this "Tafel" region signifies that different reactions are controlling the rate. This conclusion will be tested in further measurements.

In the second group of data, shown in Figure 4, the effect of incubation of the B. pasteurii in urine is considered. Here the effect of an 18-hour incubation was measured. Indications are that when the B. pasteurii is incubated, higher limiting current densities result, but that the same reaction is rate controlling, as indicated by the essentially parallel linear sections of the curves. It is apparent that over the entire curve the cell with the incubated B. pasteurii provides increased current for the same potential. For example, at 170 mV, the cell with incubated B. pasteurii carried 0.15 mA/cm^2, whereas only 0.01 mA/cm^2 was carried by the cell to which B. pasteurii had been without incubation. The advantage of allowing time for the growth of B. pasteurii is apparent.

4.8.2 Continuous Electrochemical Cell Studies

A cell was constructed for use in studies of the continuous production of power from urine for long periods of time. The cell was designed so that material and energy balance data could be obtained for various biochemical systems under sterile conditions.

Experiment I

Initial tests were run by maintaining a platinized platinum anode immersed in the B. pasteurii culture at a constant potential of +0.15 V vs the S.C.E. The cell was operated for 7 days at varying

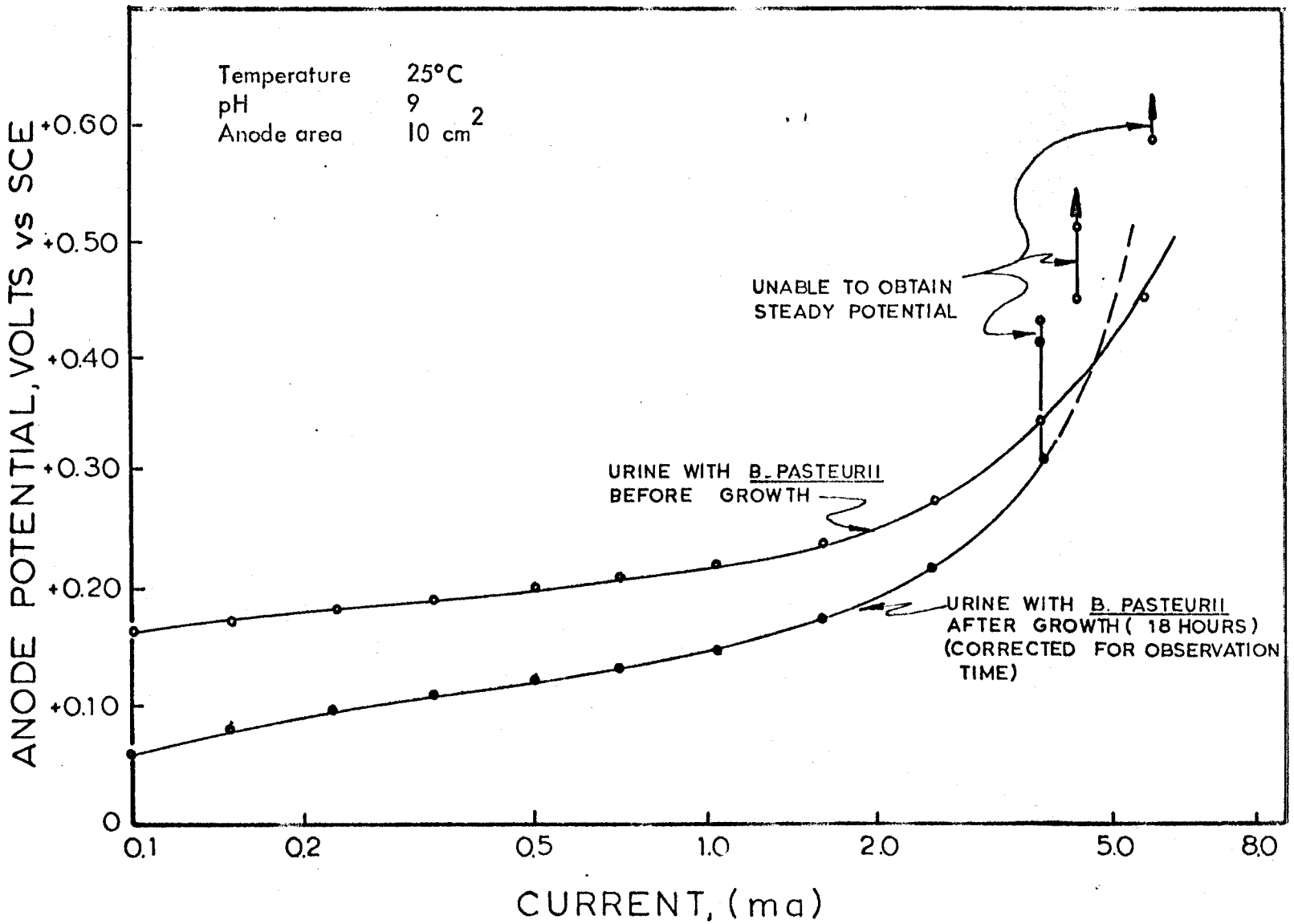


FIGURE 4: Anodic Polarization of Platinum in Urine, Effect of B. pasteurii growth

flow rates. A steady current of approximately 0.023 mA/cm^2 was maintained for several days. The maximum current obtained was about double this value. The current was responsive to feed rate, increasing with increased feed rate of urine. Deposition of insoluble salts on the electrode caused severe polarization and made it necessary to terminate the run.

Experiment 2

The problems created by salt precipitation necessitated the addition of EDTA to the urine despite the objections raised earlier. It was found that current densities at an anodic potential of +0.15 V vs S.C.E. were approximately 0.046 mA/cm^2, twice that observed previously in cultures without EDTA. This activity was maintained for more than 4 days.

The low current densities exhibited during these experiments are consistent with data obtained under Contract DA 36-039 SC-90866, during which it was shown that black platinum is not an effective electrode catalyst for ammonia. The maximum current densities obtained with black platinum anodes were low (3 mA/cm^2 or less) and the performance of such electrodes decreased rapidly with time because ammonia severely poisons the black platinum surface. Consequently, until a more suitable catalyst can be found, the ammonia anode is not, at this time, a feasible approach to producing practical amounts of power.

4.8.3 Attachment of Bacteria to Electrodes

4.8.3.1 Compression-type Electrode

Improvement of the potential-current parameters of the cell would be expected if advantage were taken of an electrode developed

under Contract DA 36-039 SC-90866. Operating on the premise that higher current densities could be obtained by confining the biological agent at the electrode surface, and thus reducing the diffusion path of the electroactive species, a compression-type electrode was constructed. The biological agent was coated onto the electrode surface by mixing into a thick paste of carbon black, urea and resting cells of the biological agent (B. pasteurii) moistened with tris buffer, pH 8.0. Polarization of this system showed an open circuit potential of -0.14 volt vs S.C.E. and a limiting current density of 1.4 mA/cm^2 (Figure 5). This represents a considerable increase in the limiting current density over that obtained at an ordinary electrode (0.2 mA/cm^2). Because of these results, it appears that the compression cell merits further study.

4.8.3.2 Filtration-Type Electrode

A preliminary study was performed to examine additional methods of attaching bacteria to electrode surfaces. A sintered gold electrode was placed in a Sietz filter and a culture of B. pasteurii was filtered through the electrode. Since the pores of the electrode were smaller than one micron, the bacteria were retained largely on the surface. The electrode was then placed directly onto Urea agar contained in a petri dish. This procedure permitted diffusion of nutrients through the pores of the electrode to the surface where the bacteria were concentrated. After a 24-hour incubation period at 30°C, the surface of the electrode was covered with a thin film of bacteria. The electrode was immersed in water and gently agitated with magnetic stirring. The film of bacteria began to slough off the electrode until, after several hours, no visible growth was evident. Microscopic examination of scrapings from the electrode surface revealed some bacteria, but these were only a fraction of the number observed before immersion in water. These results demonstrate that although this technique for initially attaching bacteria to the surface of an electrode appears to be feasible, further studies will be required to retain the organisms on the

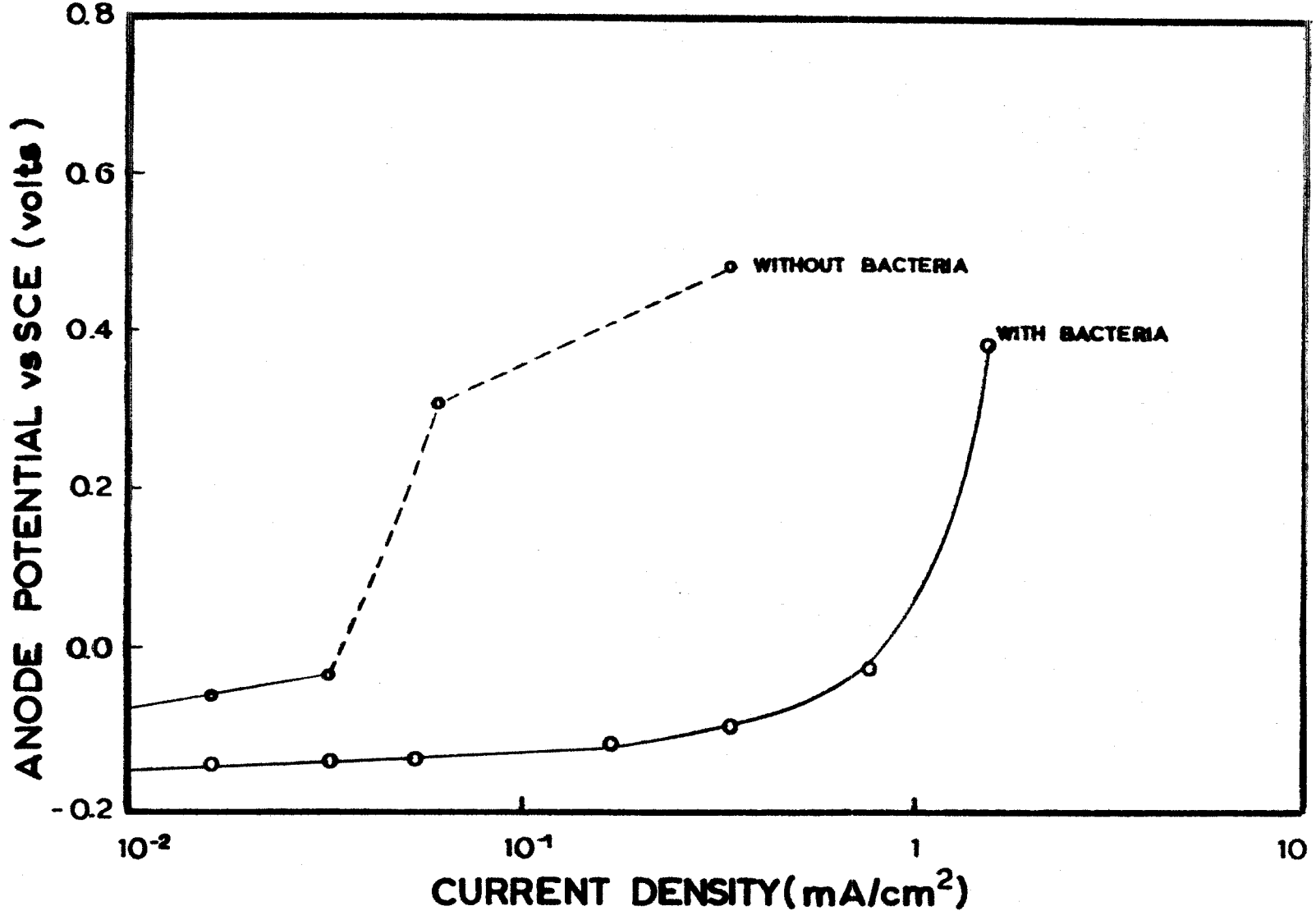


FIGURE 5

Current-Potential Curve of a Compression-Type Electrode using the Urea - B. pasteurii System

electrode in an aqueous environment, particularly if agitation is present. One approach would be the use of some conductive material which would firmly adhere the bacteria to the electrode without interfering with diffusion.

4.9 Electrochemical Control of E_h

An exploratory program was begun late in the program to determine the feasibility of using an anodically polarized electrode along with oxygen in an activated sludge culture to "poise" the redox potential of the solution at oxidizing values (positive) independent of oxygen transfer rates.

A significant portion of the power requirements in the activated sludge process is used for agitation involved in maintaining oxygen saturation of the waste materials. Approximately 10 times the oxygen actually metabolized by the microorganisms during waste degradation is fed to the waste materials for this purpose.

Without expenditure of this oxygen to maintain oxidizing conditions, the efficiency and quality of the waste treatment is much poorer due to the ability of the microorganisms to metabolize in the absence of oxygen by anaerobic pathways. Anaerobic metabolism is slower than aerobic metabolism and yields by-products which are undesirable in a closed system. It is contended that much more efficient and consequently less power-consuming maintenance of aerobic conditions can be effected by electrochemical means.

It is probable that small quantities of a redox mediator, such as methylene blue, will be required to aid in contacting the electrode with the solution.

As the first step in this study, a test cell was designed and fabricated for the purpose of examining the influence of an anodically polarized carbon electrode on the metabolism of the yeast, Saccharomyces cerevisae. The object of the experiment was to determine whether yeast could be prevented from metabolizing anaerobically in the absence of oxygen by electrochemically maintaining or poising the redox potential of the medium in an oxidizing region. Yeast was selected as a test organism because

it metabolizes both aerobically and anaerobically but produces ethanol only anaerobically. Thus, the yeast culture could be grown aerobically with glucose, placed in an anaerobic anode compartment of the cell before the nutrients were exhausted, and examined for evidence of ethanol production while the redox potential of the culture was being maintained at oxidizing values by a redox mediator maintained at a fixed potential by the carbon anode. The lack of ethanol production under these conditions would constitute a successful first step in this study.

A cell was constructed but, unfortunately, due to the late introduction of this study in the program, time did not permit obtaining any significant results.

4.10 Experimental Procedures

4.10.1 Sources of Urine and Feces

Urine and feces for this program were obtained from volunteers. Magna Corporation male contributors were asked to consume a simulated space diet currently under study elsewhere (141). The principal feature of this diet was omission of raw fruits and vegetables, which are abundant in cellulose. Personnel on otherwise specialized diets or who were taking medications were asked not to contribute.

A total of approximately 20 gal of urine and 30 lb of feces was collected. Both were frozen immediately after collection. At the close of the collection program, the urine was thawed and mixed, distributed in approximately half-gallon lots, and refrozen.

The procedure used to process feces consisted of freeze-drying the feces without thawing, breaking the freeze-dried material down to a powder in a Waring blendor, compositing and storing under nitrogen in a refrigerator.

A Thermovac Freeze-Dry Apparatus, Model FD-3 (Thermovac Industries Corp., Copiague, N.Y.) was used to lyophilize the feces. Lyophilization was considered to be the most suitable method of protecting the components of feces from biological degradation for prolonged periods.

The lyophilized feces composite was submitted for analysis to Truesdail Laboratories, Inc., 4101 N. Figueroa St., Los Angeles 65, Calif. The results are shown in Table 22. Note that carbohydrate content is deceptively high since this value was obtained by difference, not analysis. Similarly, "protein" was derived from nitrogen content only and not by actual protein analysis.

TABLE 22

Analysis of Composited Feces

Moisture	2.57%
Cellulose (crude fiber)	3.34%
Total protein (n x 6.25)	34.33%
Total lipids (ether extract)	18.0%
Carbohydrates (by difference)	29.26%
Ash	12.50%
Total invert sugar before inversion	2.12%
Total invert sugar after inversion	2.12%

Hydrolysis was used to determine lipids and sugar after inversion

4.10.2 Sources of Cultures

1. Sarcina ureae - The National Collection of Industrial Bacteria, Aberdeen, Scotland (No. 8691).
2. Micrococcus ureae - The Institute for Fermentation, Osaka, Japan (No. 3767).
3. Lactobacillus bifidus var. ureolyticus - Dr. R. N. Doetsch, University of Maryland, Md.

4. Brevibacterium ammoniagenes - The National Collection of Industrial Bacteria, Aberdeen, Scotland (No. 8143).

5. Bacillus pasteurii - The American Type Culture Collection, Washington, D. C. (No. 11859).

4.10.3 Screening Program-Ureolytic Organisms

Sarcina ureae

Urea broth consisted of Difco nutrient broth, 10 g; Difco yeast extract, 5 g; distilled water, 1 liter. After cooling, 100 ml of 20% urea (filter-sterilized) was added. Final pH was 8.0. The medium was inoculated with a 1% inoculum and cultured aerobically and anaerobically at 30°C for 24 hours. Turbidity (optical density) was measured in a Beckman DU spectrophotometer using incident light of 660 mμ wavelength. Growth (OD_{660} 0.95, 1/4 dilution) and ammonia production as evidenced by odor occurred only in the aerobic culture.

Urea broth agar plates were streaked with a culture of S. ureae and incubated aerobically at 30°C and anaerobically under hydrogen gas in an anaerobic jar for 24 hours. Again, growth was evident only aerobically.

Micrococcus ureae

The bacterium was received as an agar slant culture and inoculated into Urea broth; subsequent growth was streaked on to Urea broth agar plates to determine purity, since the slant appeared to be contaminated. Several types of colonies were observed. Isolated colonies were picked, inoculated into Urea

broth and growth was again streaked on to agar plates. Although this procedure was repeated several times, isolation of M. ureae was not achieved. In fact, even the original contaminated culture did not exhibit ureolytic activity.

Lactobacillus bifidus var. ureolyticus

The bacterium was subcultured repeatedly anaerobically at 37°C in the following medium based upon the recipe used by Gibbons and Doetsch (43); Difco yeast extract, 3.0 g; Difco nutrient broth, 10.0 g; K_2HOP_4, 3.0 g; KH_2PO_4, 3.0 g; glucose, 5.0 g; Na_2CO_3, 3.0 g; resazurin, 0.001 g; sodium thioglycollate, 1.0 g; distilled water, 1 liter. 3.0 g urea (filter-sterilized) was added to the cooled basal medium; final pH, 7.0. Excellent growth occurred within 24 hours but the odor of ammonia was not detected. Culturing in (1) same medium containing 20% urea, (2) Urea broth, and (3) urine failed to stimulate ureolytic activity.

Bacillus pasteurii

Cultures were grown in Urea broth as described earlier for S. ureae. After 24 hours incubation, the OD_{660} of the aerobic culture was 0.38 (1/4 dilution) compared to 0.12 (1/4 dilution) with anaerobic growth. The odor of ammonia was strongly evident in both cultures. With anaerobic growth in urine, the odor of ammonia was evident after 24 hours. The concentration of urea was reduced from 9.0 mg/ml to 0.24 mg/ml, a decrease of 97.3%.

Brevibacterium ammoniagenes

Anaerobic and aerobic growth studies with Urea broth and urine were performed in a manner similar to that described for

S. ureae. The pH of Urea broth and urine was adjusted to 7.4 and the cultures incubated at 30°C. Good growth and ammonia production was noted after 24 hours aerobic growth in Urea broth but not until from 48 hours to 5 days under anaerobic conditions. After 5 days anaerobic growth in Urea broth, the concentration of urea was reduced from 18.5 mg/ml to 11.65 mg/ml, a decrease of 37%.

Ureolytic activity was observed after 48 hours of aerobic growth in urine but not until after 6 days of anaerobic growth. In the anaerobic urine culture the concentration of urea was reduced from 10.75 mg/ml to 5.8 mg/ml, a decrease of 46%.

4.10.4 Optimization of Ureolytic Activity

4.10.4.1 Analytical Methods

A 1% inoculum of a culture of B. pasteurii was added to 50 ml of urine containing 0.1% ascorbic acid. The initial pH was adjusted to 8.8. Ten ml aliquots of the inoculated urine were dispensed into 3 125-ml Warburg respirometer flasks. Mercury was used as the manometer fluid. Appropriate precautions were taken to prevent contamination. The flasks were evacuated and flushed with heliumwhile being agitated in a 30°C water bath. After the flasks were flushed for 30 minutes, the manometers were adjusted to atmospheric pressure and the flasks were incubated for 4 days.

The Warburg respirometers were not calibrated and, therefore, approximate K values were used based upon previous calibrations.

4.10.4.2 Storage of Urine

Anaerobic growth of fresh, refrigerated (4 days), and thawed frozen urine from the same donor was compared. 0.1% ascorbic acid was added to

all urine. The pH was adjusted to 8.4 and a 1% inoculum of B. pasteurii was used. Duplicate 30-ml screw cap test tubes were filled to capacity, and incubated at 30°C.

4.10.4.3 pH

Duplicate 30-ml screw cap test tubes were filled to capacity with urine (0.1% ascorbic acid) inoculated with 1% inoculum of B. pasteurii. The initial pH of duplicate cultures was adjusted to pH 7.5, 8.0, 8.5, 9.0 and 9.5 with NaOH and incubated at 30°C.

4.10.4.4 Temperature

300 ml urine containing 0.1% ascorbic acid was inoculated with 1% of a culture of B. pasteurii. The pH was adjusted to 8.6. Duplicate 30-ml screw cap tubes were filled to capacity and incubated at 25, 30 and 35°C for 24 hours.

4.10.4.5 Vitamins

The basal medium consisted of urine (0.1% ascorbic acid), pH 8.5. Nicotinic acid, thiaminehydrochloride and biotin were added to the basal medium to produce the following final concentrations of the respective vitamins in urine: (1) nicotinic acid (0.5 µg/ml), (2) thiamine hydrochloride (0.5 µg/ml), (3) biotin (0.1 m µg/ml), (4) nicotinic acid (0.5 µg/ml), and biotin (0.1 m µg/ml), (5) nicotinic acid (0.5 µg/ml) and thiamine hydrochloride (0.5 µg/ml), (6) biotin (0.1 m ug/ml) and thiamine hydrochloride (0.5 µg/ml), (7) nicotinic acid (0.5 µg/ml), thiamine hydrochloride (0.5 µg/ml) and biotin (0.1 m µg/ml). The control contained no added vitamins. Duplicate 30-ml screw cap test tubes were filled to capacity with the various media preparations,

inoculated with a 1% inoculum of a culture of B. pasteurii and incubated at 30°C. The nicotinic acid was obtained from Eastman Organic Chemicals, Rochester, New York; biotin from California Corporation for Biochemical Research, Los Angeles, California; thiamine hydrochloride from Hill Drugs, Anaheim, California.

4.10.4.6 Addition of Urea to Urine

Experiment 1

100 ml aliquots of urine containing 1.4 (normal urine) and 3.1% urea and 0.1% ascorbic acid were inoculated with 1% of a culture of B. pasteurii. Urine was supplemented with filter-sterilized urea. The pH was adjusted to 8.5. 30-ml screw cap tubes were filled to capacity and incubated for 24 hours at 30°C.

Experiment 2

100 ml aliquots of urine containing 1.0 (normal urine) 1.9, 2.7, 3.5 and 3.9% urea and 0.1% ascorbic acid were inoculated with 1% of a culture of B. pasteurii. Urine was supplemented with filter-sterilized urea. The pH was adjusted to 8.6. 30-ml screw cap tubes were filled to capacity and incubated for 24 hours at 30°C.

4.10.4.7 Addition of Feces to Urine

Experiment 1

The media were prepared by adding 4, 20 and 50% frozen whole feces (by weight) to filter-sterilized urine. The feces was

sterilized by autoclaving. The media were inoculated with 1% of a urine-grown culture of B. pasteurii and the pH adjusted to approximately 8.5. Duplicate 30-ml screw cap test tubes were filled to capacity with the inoculated media and incubated at 30°C for 7 days.

Experiment 2

The medium consisted of sterile urine to which was added 1, 5 and 10% lyophilized feces (by weight). The feces was sterilized by autoclaving. The medium was inoculated with 1% of a urine-grown culture of B. pasteurii and the pH adjusted to 8.8. Control cultures were identical but lacked feces. Duplicate 30-ml screw cap test tubes were filled to capacity with the inoculated media and incubated at 30°C for 4 days.

Experiment 3

The procedures were identical to those in Experiment 2 with the exception that the inoculum consisted of a culture of B. pasteurii which had been subcultured 5 times in a 5% feces in urine medium.

4.10.4.8 Continuous Culture

The continuous culture apparatus is illustrated in Figure 6. Sterile urine was fed into the growth vessel by means of a Harvard Infusion-Withdrawal pump, Model 600-950 (Harvard Apparatus Company, Inc., Dover, Massachusetts). The capacity of the growth vessel was 100 ml and the culture was mixed with a magnetic stirring bar placed on the bottom of the growth vessel. The growth vessel was immersed in a recirculating constant temperature water bath maintained at 30°C.

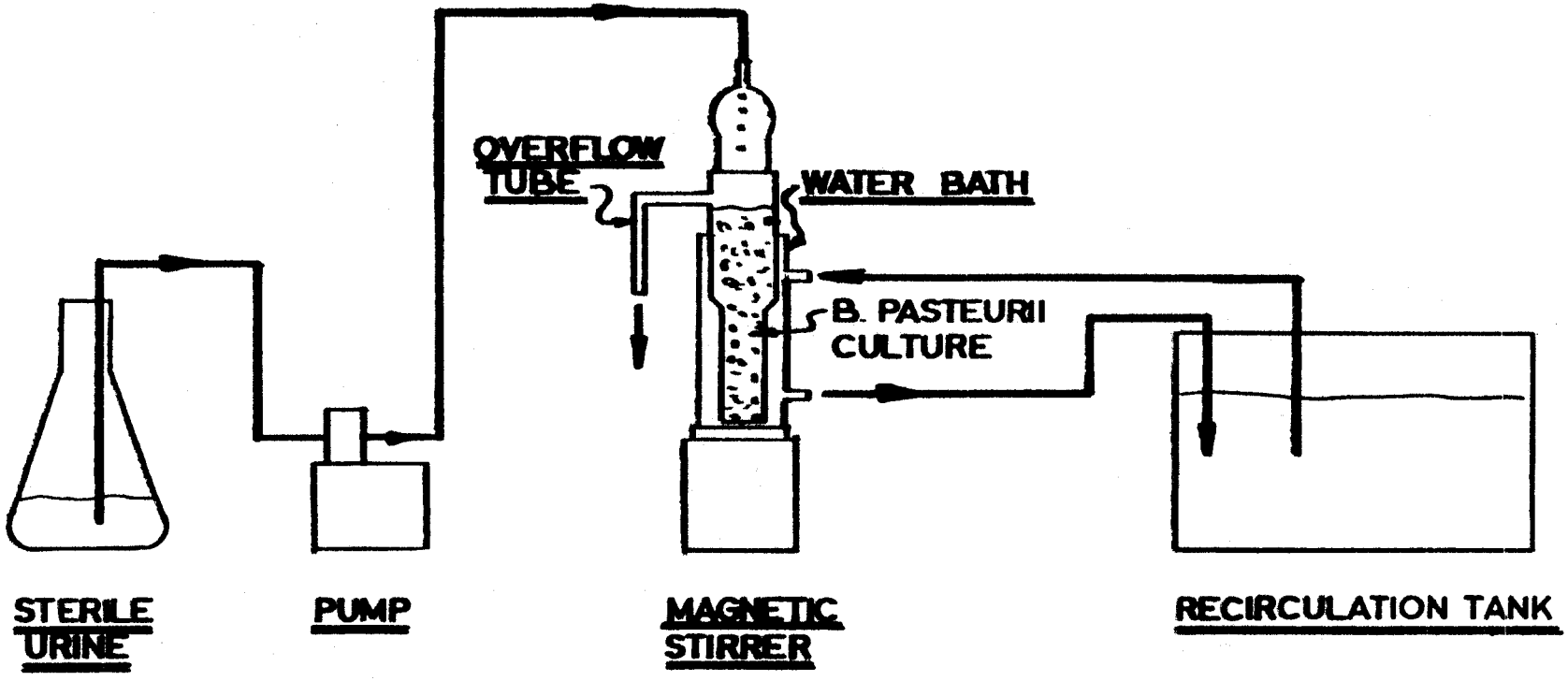


FIGURE 6

Schematic of Continuous Culture Apparatus

Samples of cultures for urea and ammonia analyses were collected directly from the growth vessel by means of a sliding glass tube which was held above the liquid line when not in service. In Experiment 1, however, samples were collected from the overflow.

The scrubbing device consisted of a 1" x 1/2" rectangular pad of polyurethane that contained a magnetic stirring bar in its center. The scrubber was maintained inside the growth vessel, above the culture, and manipulated from the outside with a horseshoe magnet.

4.10.4.9 Addition of EDTA to Urine

Experiment 1

Sterile urine containing various amounts of EDTA (disodium ethylenediamine-tetraacetate) was adjusted to pH 8.2 with NaOH. It was found that the addition of 0.15% EDTA completely prevented the characteristic precipitation which normally occurs at this pH. As a result of these tests, urine containing 0.10% and 0.15% EDTA was inoculated with 1% of a B. pasteurii culture and dispensed into 30-ml screw cap tubes. Controls were identical but lacked EDTA. The cultures were inoculated at 30°C and inspected daily for the odor of ammonia.

Experiment 2

Aliquots of urine were supplemented with 0.2% EDTA, inoculated with a 1, 5 and 10% inoculum of B. pasteurii and dispensed into duplicate 30-ml screw cap test tubes. Control cultures were inoculated identically, but lacked EDTA. The final pH was 8.8.

4.10.5 Feces Studies

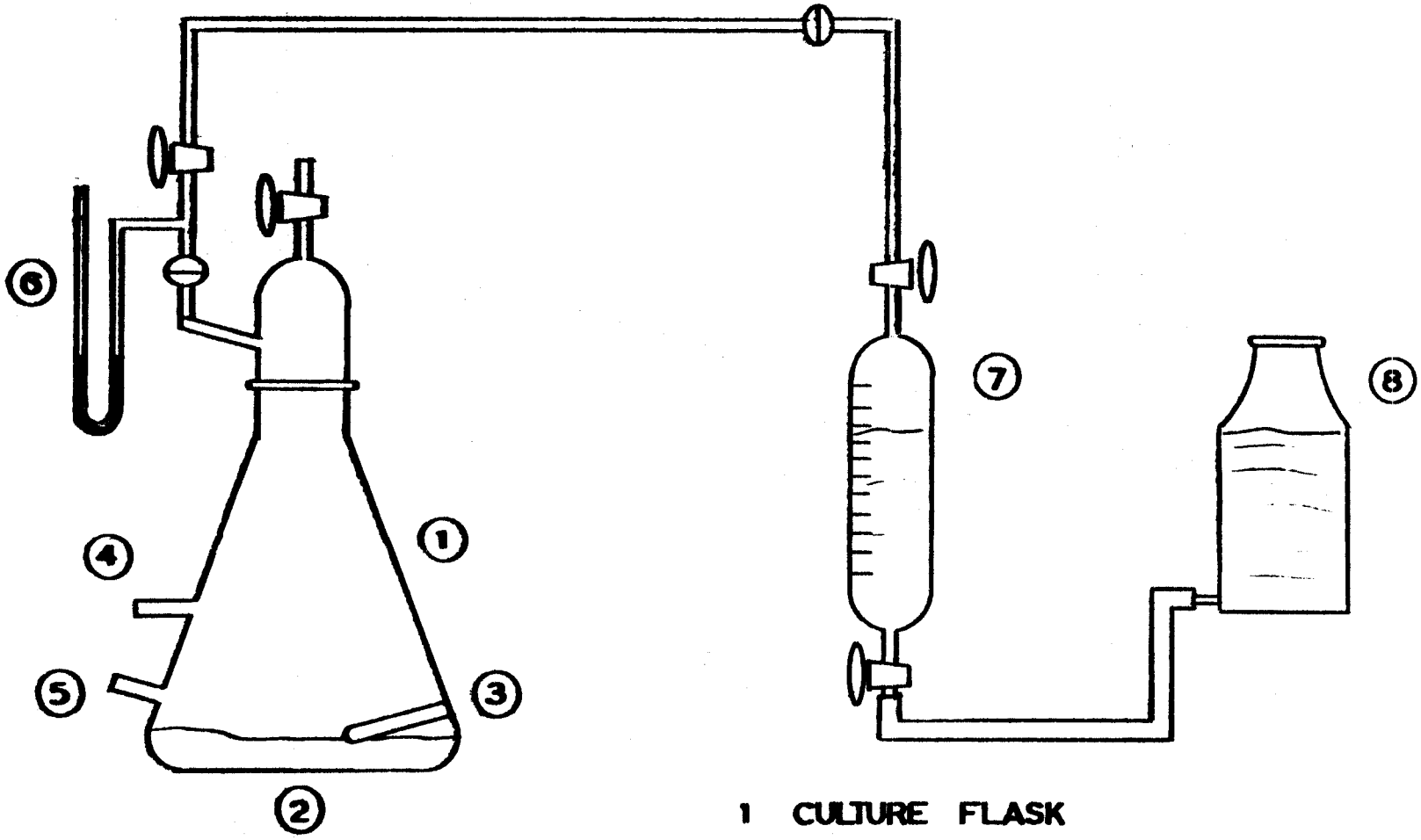
4.10.5.1 Hydrogen Production from Feces

Experiment 1

Treated sewage was obtained from the Orange County primary sewage treatment plant at Huntington Beach, California. Three-liter macrorespirometers (Figure 7) were used as digestors. Digestion flasks contained 200 ml of inoculated medium prepared from 100 ml potassium acid phthalate-NaOH buffer (pH 5.0), 25 ml sterile urine, 5 g heat-sterilized lyophilized feces and 375 ml treated sewage. Control flasks contained 200 ml of inoculated medium prepared from 100 ml potassium acid phthalate-NaOH buffer (pH 5.0), 25 ml sterile distilled water and 375 ml treated sewage. The pH of both cultures was adjusted to 5.5. Flasks were evacuated and flushed 10 times with helium to displace air. Manometers were filled to capacity with acidified water. The digestors were incubated at 30°C with shaking for 6 days. Total and volatile solids were determined on original and final cultures by standard methods (142). Gas evolution was measured manometrically and the gas phase analyzed using a Beckman GC 2 gas chromatograph.

Experiment 2

The feces-adapted sewage culture was obtained from Dr. C. Golueke, Sanitary Engineering Research Laboratory, University of California, Berkeley, California. 150 ml of the culture was added to 40 ml potassium acid phthalate-NaOH buffer (pH 5.0),



MACRORESPIROMETER

FIGURE 7

1 CULTURE FLASK
2 BACTERIAL CULTURE
3 THERMOMETER WELL
4 GAS SAMPLING PORT
5 CULTURE SAMPLING PORT
6 MERCURY MANOMETER
7 500 ML MANOMETER
8 LEVELING BOTTLE AND ACIDIFIED H_2O

10 ml sterile urine and 18.6 g heat-sterilized lyophilized feces. 50 ml of this mixture were dispensed into three 250-ml macrorespirometers. 0.5 gm filter-sterilized glucose was added to 2 of the 3 cultures. Final pH was adjusted to 5.8. The macrorespirometers were evacuated and flushed 10 times with helium and incubated at 30°C. Gas evolution was measured manometrically and the gas phase was analyzed by means of gas chromatography.

Experiment 3

The medium consisted of 100 ml sterile potassium acid phthalate-NaOH buffer (pH 5.0), 100 ml sterile urine and 20 g heat-sterilized lyophilized feces which was inoculated with 2 ml of an anaerobic culture of E. coli grown for 24 hours in Difco Heart Infusion Broth. 50 ml of the inoculated medium was dispensed into each of two 250-ml macrorespirometers. Two cultures contained 0.5 ml filter-sterilized glucose. Final pH was 6.8. Subsequent procedures were identical to those described in the preceding experiment.

4.10.5.2 Enzymatic Hydrolysis of Fecal Components

1. Cellulase

Assay

A standard curve was prepared using glucose solutions containing weighed amounts of glucose from 0.05 mg/ml to 1.0 mg/ml. The data obtained gave a satisfactory linear relationship between optical density and glucose concentration in the range of 0.1 mg/ml to

1.0 mg/ml. However, at concentrations lower than 0.1 mg the absorption (at 540 mμ) of the developing compound (3,5-dinitrosalicylic acid) was greater than that of the brown reaction product.

A preliminary assay of the crude cellulase preparation (Nutritional Biochemical Corporation, Cleveland, Ohio) indicated that it possessed some cellulase activity (0.08 mg glucose/hr/mg dry enzyme prep.) and a fair amount of reducing sugar contaminants. The zero time sample taken from the cellulose-cellulase reaction mixture (one ml cellulase solution) gave a color equivalent to 3.0 mg of glucose. A check of both the cellulase and cellulose solutions clearly demonstrated that the reducing compounds were contained entirely in the cellulase preparation. A protein (143) determination (Lowry) of the cellulase solution indicated that there was slightly less than 0.1 mg protein/ml.

Dialysis Procedure

100 mg of the crude fungal cellulase preparation (N.B.C.) was suspended in 50 ml of distilled water. This solution was placed into cellophane dialysis tubing and allowed to remain in 0.01 M phosphate buffer, pH 7.0 at O°C for a period of 16-18 hours. The enzyme solution was removed from the dialysis tubing and diluted to 100 ml with cold distilled water.

Temperature Study

A 1% solution (w/v) of carboxymethylcellulose (Hercules Cellulose gum 7 HP) was diluted with 20 ml

of McIlvaine buffer solution, pH 5.0 and 60 ml of distilled water. Nine ml of this solution was added to each of 5 tubes. Each set of 5 tubes was placed into a water bath of the appropriate temperature (30°C, 40°C and 50°C) and allowed to equilibrate. To each tube one ml of the above mentioned enzyme solution was added and the mixture was well agitated. A zero time sample (one ml) was removed immediately from one tube and the amount of reducing sugar determined as described previously. Samples were taken every hour thereafter from a different tube. Five samples were taken in all. The amount of reducing sugars (glucose and cellobiose) present were determined by referring to a calibration curve (O.D. vs glucose concentration) as described previously.

2. Lipase

Lipase Assay

The lipase assay used is a manometric method described in the literature by Wills (108). In those experiments where triacetin (Eastman 256) served as the substrate, 0.85 ml of 1.0 M $NaHCO_3$, 0.1 or 0.05 ml of enzyme solution, and water (to make a total volume of 3.2 ml) were added to the main compartment of the Warburg flask. The triacetin (neutralized)solution (30 to 50 μ M) was added to the side bulb and was tipped in to start the reaction. When the 20% feces (w/v) solution (in urine) was used as the substrate, the lipase solution was tipped in to start the reaction and the feces solution (1.0 ml) was added to the main compartment along with the $NaHCO_3$ solution. In both cases, the Warburg cups were purged for

15 minutes with 100% CO_2. This concentration of $NaHCO_3$ and the 100% CO_2 atmosphere buffered at pH 7.4. All manometric assays were carried out at 37°C.

Lipase Solution

One hundred milligrams of crude pancreatic lipase (Lipase 448-N.B.C.) was suspended in 10 ml of distilled water, placed in a cellulose dialysis bag and dialyzed against 0.01 M phosphate buffer, pH 7.0 for 18 hours. Particulate material was removed by cold (4°C) centrifugation and the enzyme solution stored at 4°C. Protein concentration was determined by the Lowry colorimetric procedure.

Feces-Urine Solution for Lipase Experiments

Twenty grams (wet weight) of whole feces was sterilized by autoclaving in the container section of a Waring blender. After cooling, 100 ml of filter-sterilized urine was added and the mixture blended for 15 minutes. The pH of the mixture was adjusted to 7.4 with 0.1 M NaOH and then stored at 4°C in a sterile flask.

4.10.6 Electrochemical Evaluation of B. pasteurii in Urine

4.10.6.1 Half-Cell Studies

The half-cells were 500-ml, five-neck flasks, separated by a cation membrane. The catholyte was 0.1 N KCl and the cathode was

platinized platinum. The anolyte was stirred with a Mag-mix and anaerobic conditions were maintained by a nitrogen purge. The temperature was not controlled. The potential was obtained with a standard calomel electrode placed in contact with an agar bridge immersed in the urine. A constant potential between SCE and anode (platinized platinum, 10 cm^2) was maintained with a potentiostat. No buffer or other solutions were used. The anode was positioned so that both faces were parallel to the current flow through the membrane. The agar bridge was positioned behind the anode (away from the membrane). An internal resistance at 175 ohms was found.

4.10.6.2 Continuous Electrochemical Cell Studies

The cell design, shown in Figures 8, 8A and 8B, consists of 6 separate parts. The glass tubing has been fitted so as to minimize the use of rubber tubing. The anode and cathode half-cells are essentially identical, being designed in the shape of an L with an "O" ring joint (65/40) at the top and side. When assembled, they form a U cell having an internal diameter of 40 mm (1-9/16"). A membrane (cation, Nepton CR-61, Ionics, Inc.) is clamped between the two half-cells to separate the electrolytes. The design is such that the membrane has a greater diameter than the electrodes, permitting the two electrodes to face each other directly. This arrangement minimizes the distortion of the current flow in the cell.

The test and auxiliary electrodes are perforated platinized platinum foil with a calculated area of 17.7 cm^2. They are spaced 4 inches apart. The internal resistance with a urine analyte and a 1 M KCl catholyte has been measured at 24 ohms.

Since the reference electrode is a saturated calomel electrode which cannot be sterilized, a glass tube containing an agar solution is used to provide a bridge between the reference electrode and urine solution. In normal operation, the capillary tip of the bridge is positioned near the

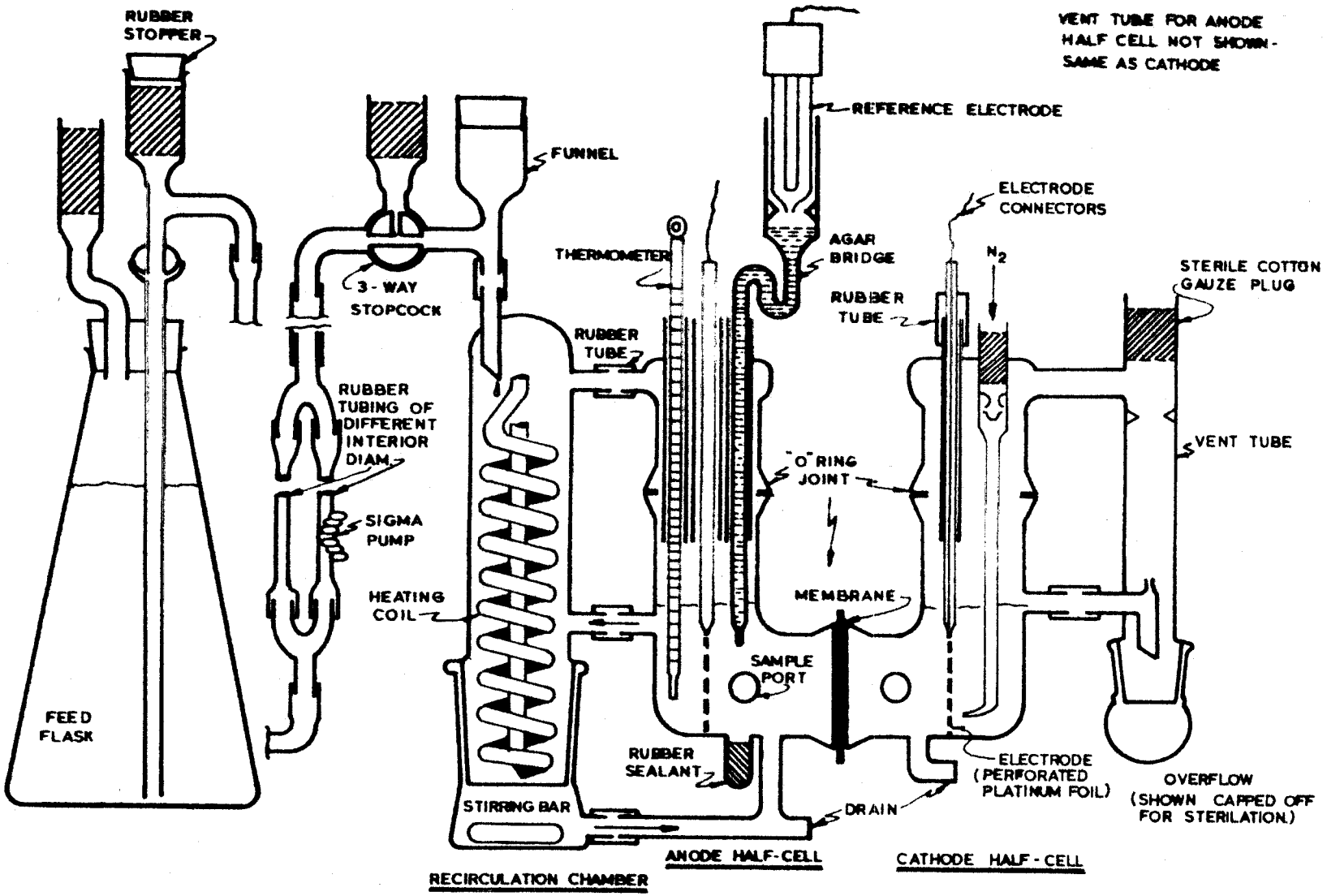


Figure 8: Cell for Electrochemical Evaluation of Urine Cultures

Figure 8-A Cell for Electrochemical Evaluation of Zinc Chloride Battery

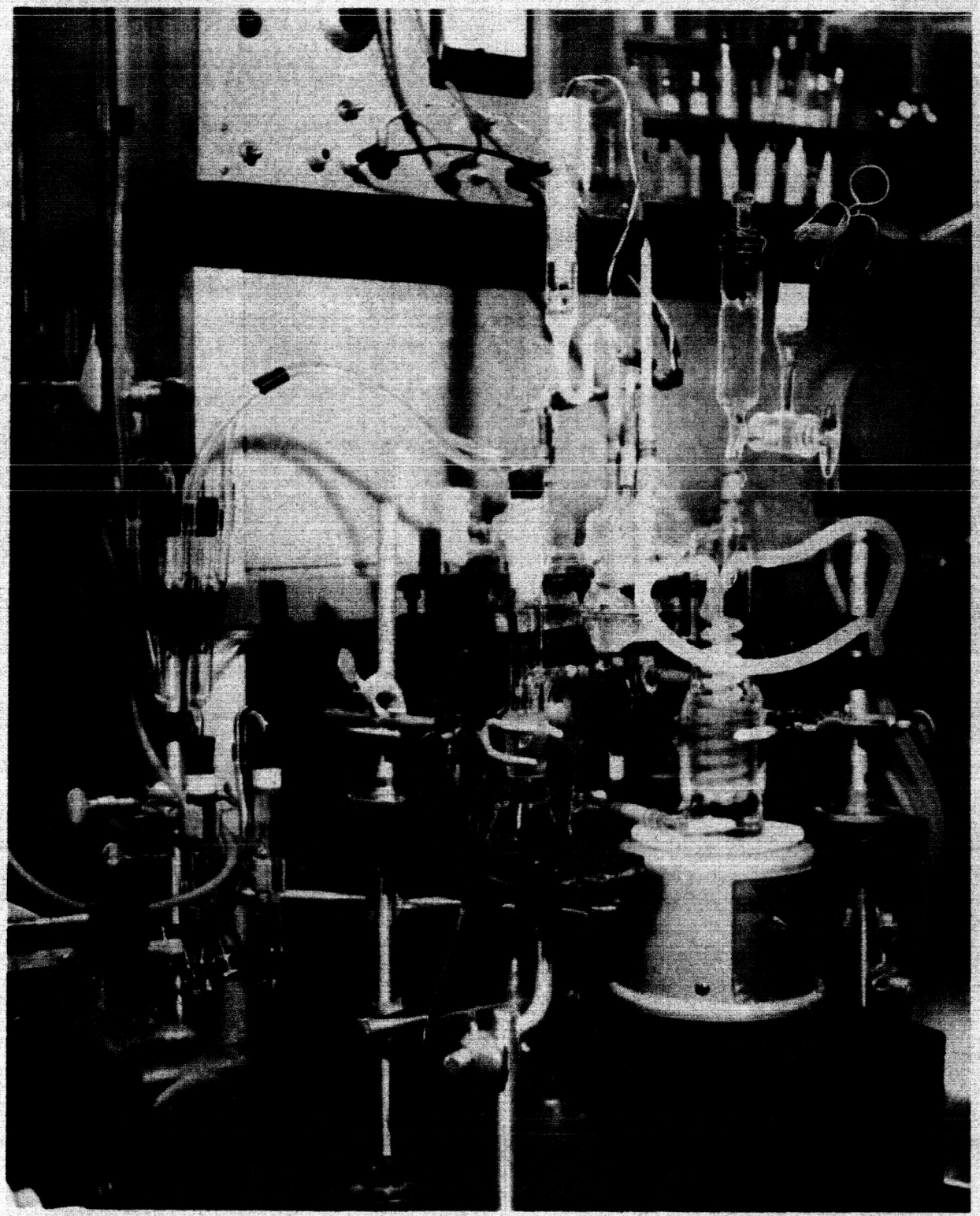


Figure 8-B Cell for Electrochemical Evaluation of Urine Cultures

surface of the anolyte so as to minimize disturbance at current distribution. During sterilization, the bridge is lowered until the capillary tip seats into a rubber sealant (Silastic RTV 731, Dow Corning), in a sealed glass tube at the bottom of the anode half-cell. This prevents the agar solution from draining out of the tube when it becomes liquid during sterilization.

The upper part or cap of each half-cell is clamped to the top "O" ring joint. In the anode half-cell, provision has been made for a thermometer, the test electrode, and the agar bridge, by using glass tubing through which each can be inserted. The cathode half-cell cap has openings for an auxiliary electrode, for purge gas (used to dilute and purge hydrogen as well as for agitation), and for liquid feed.

The vent tube in the cap of each half-cell is plugged with sterilized cotton. An overflow tube in the bottom of the half-cell is connected with rubber tubing to a continuation of the overflow in the vent tube. During operation, the flask which has been clamped to the vent tube to collect the overflow is thus cross-pressurized to the half-cell, and both are sealed from direct contact with the atmosphere by the sterile cotton plug.

The anode half-cell is heated and agitated by use of an agitation chamber consisting of two parts. The bottom part has a side outlet which can be connected to an inlet in the anode half-cell. The upper part is vented to the anode half-cell and contains a return line for recirculated anolyte. Fresh urine is fed into this recirculation chamber for mixing prior to contacting the electrode. The recirculated anolyte passes over a glass coil through which water contained at a constant temperature is circulated. The total volume of anolyte is 215 ml ($\pm$ 5 ml).

Fresh anolyte is fed to the cell by means of a Sigma pump equipped with a calibrated Zero-Max variable speed drive. The unit was modified by inserting a speed reducer (Alling-Lander, 50/1) between the Zero-Max and pump. A flow rate of from 1 to 42 ml/hr can be obtained. The feed tube is connected to the agitation chamber through a three-way stopcock. A

funnel is also provided as an integral part of the feed line for sterile liquids which may be required during operation. The feed tube connects to the feed flask by means of a ball joint so as to facilitate changing feed flasks without undue interruption of flow or chance of contamination.

Both half-cells are equipped with sample ports and drains stoppered with rubber serum caps. The cells have legs made of glass rod so that when assembled the unit stands level. In practice the assembly is wired to a board to which is affixed aluminum rods so that clamps can be used to take the weight of the overflow flasks.

The entire assembly, using sealed caps in place of overflow flasks, can be sterilized along with the rubber feed tubes. After sterilization, the board containing the assembled apparatus is mounted at a sufficient height to permit the overflow flasks to be properly placed below the cell assembly.

A constant temperature bath is employed to maintain the cell temperature at 30 ± 0.5°C.

Experiment 1

The cell was sterilized by autoclaving, purged with sterile nitrogen and filled with filter-sterilized urine. No evidence of contamination was observed after 2 days incubation at room temperature. The pH was then adjusted to 8.8 with sterile NaOH. A current of 0.5 to 0.20 mA (0.0085 to 0.0113 mA/cm^2) was observed over a 20 minute period at + 0.15 V (SCE vs anode). The urine was inoculated with a 1% culture of B. pasteurii and incubated at 31°C. After 2 days incubation, ammonia was evident. At this time, the anode potential (open circuit) vs SCE had dropped to -0.244 V and the pH increased to 9.2 An initial current of 2.0 mA at + 0.15 V was obtained which rapidly dropped to 0.8 mA. After 4 hours, the current had dropped to 0.6 mA where it held during the remainder of the day. The flow rate was approximately

14 ml per hour. The flow rate was reduced to 5.6 ml per hour overnight and by the next morning the current had dropped to 0.44 mA. When the flow rate was increased to 30 ml per hour, the current increased over a 24-hour period to 0.56 mA. The flow rate was decreased to approximately 9 ml per hour which resulted in a steady current of 0.40 mA over a 4-day period. The pH of the culture remained at 9.2 during the test period. The catholyte (1.0 N KCl, pH 9.3), upon analysis, was found to contain ammonia indicating that ammonia ion had diffused through the cation membrane. A sample of the culture removed from the cell at the end of the run and analyzed for ammonia was found to contain 5.9 mg NH_3 per ml, indicating good bacterial activity.

Experiment 2

Several modifications were introduced. The cation membrane was replaced by an anion membrane to prevent diffusion of ammonium ion. A device for accurately measuring flow rate was installed (Figure 9).

The cell was prepared for electrochemical measurements as described above, with the exception that 0.2% EDTA was added to the culture. The initial open circuit potential of the anode was -0.203 V vs SCE. At a current level of approximately 1.0 mA (0.056 mA (cm^2)) maintained during the 4-day test period, the anode polarized to +0.15 V vs SCE.

4.10.6.3 Attachment of Bacteria to Electrodes

The compression-type electrode was constructed as shown in Figure 10. The carbon anode was coated with a thick paste containing 500 mg of

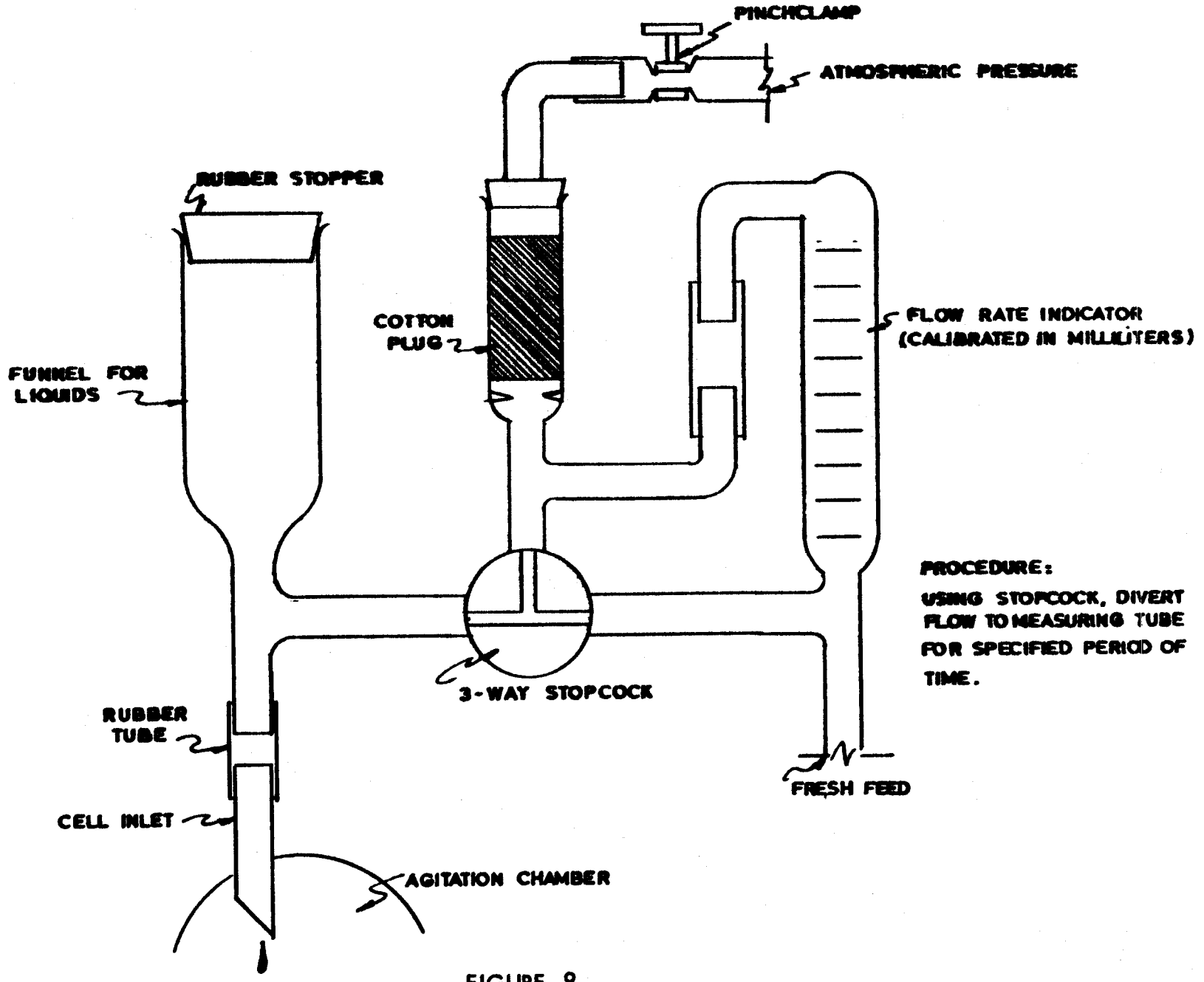


FIGURE 9

Device for Measuring Absolute Flow Rate to Urine Cell

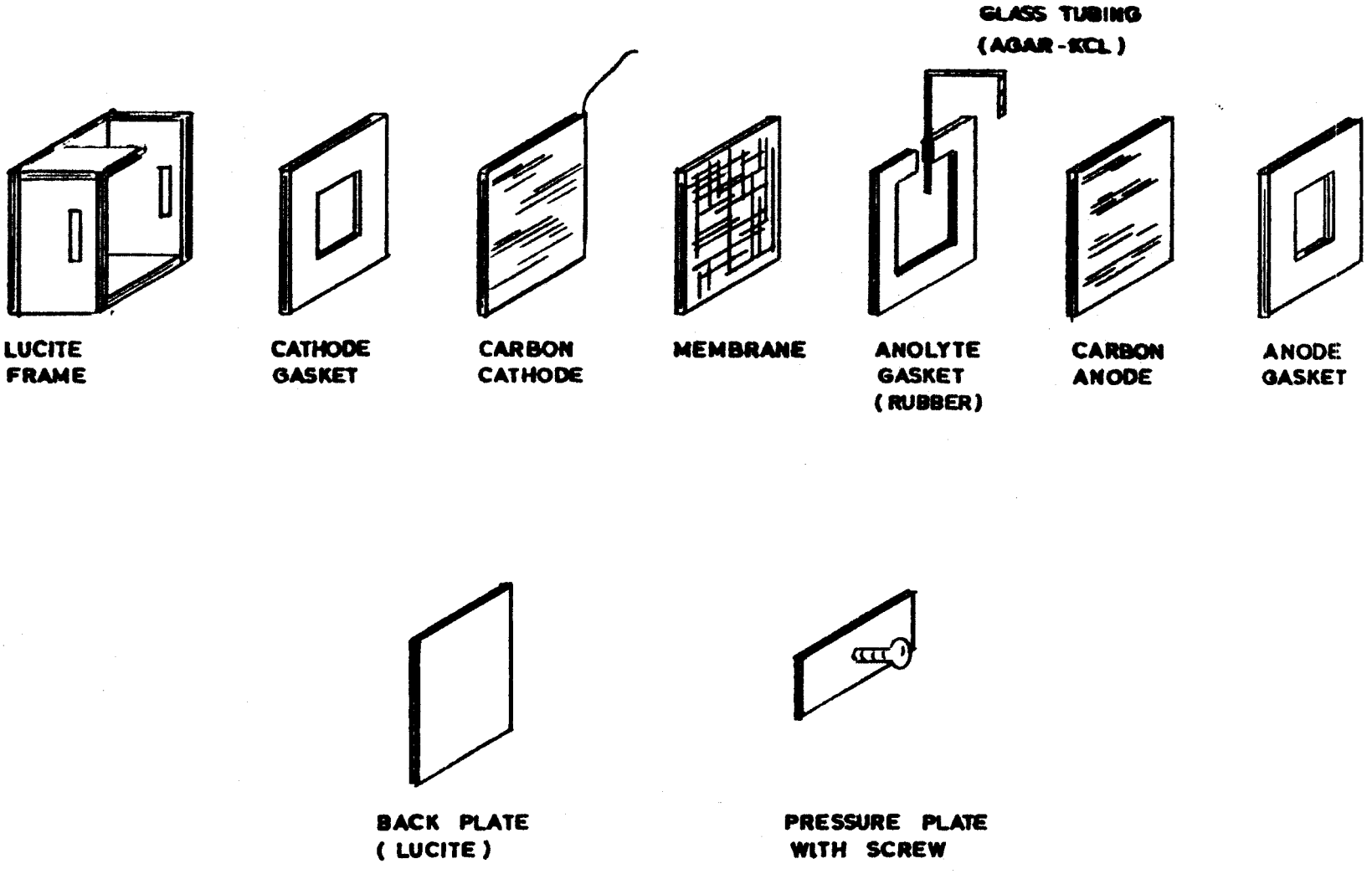


FIGURE 10

Schematic Diagram of the Compression–Type Electrode in a Cell Assembly

carbon black, 50 mg of platinum black, 100 mg of urea, and 50 mg of B.pasteurii (wet weight), mixed with sufficient tris buffer (0.1 M, pH 8.0) to make a paste. A cation-exchange membrane (Ionics, Nepton CR 61) was placed between the two electrodes. A glass tube, drawn to a capillary at one end and filled with saturated KCl in agar, was inserted into the anode paste and served as a salt bridge to a calomel reference electrode. The entire cell assembly was placed under pressure by turning a screw on the back plate, thus assuring good contact between the membrane and electrodes. Polarization measurements were made as described in Section 4.10.6.1.

4.10.7 Electrochemical Control of E_h

The cell construction for study of the electrochemical control of E_h is represented schematically in Figure 11. It consists of three compartments, an anode compartment, a cathode compartment and a center compartment. The compartments are separated by membranes to prevent interdiffusion. The anode compartment, the compartment of interest, contains a probe to measure the E_h of the solution consisting of a platinum electrode and an agar bridge leading to a reference electrode, an anodically polarized carbon electrode, an oxygen sensing electrode (Magna Corp., Santa Fe Springs, California), and a gas purge.

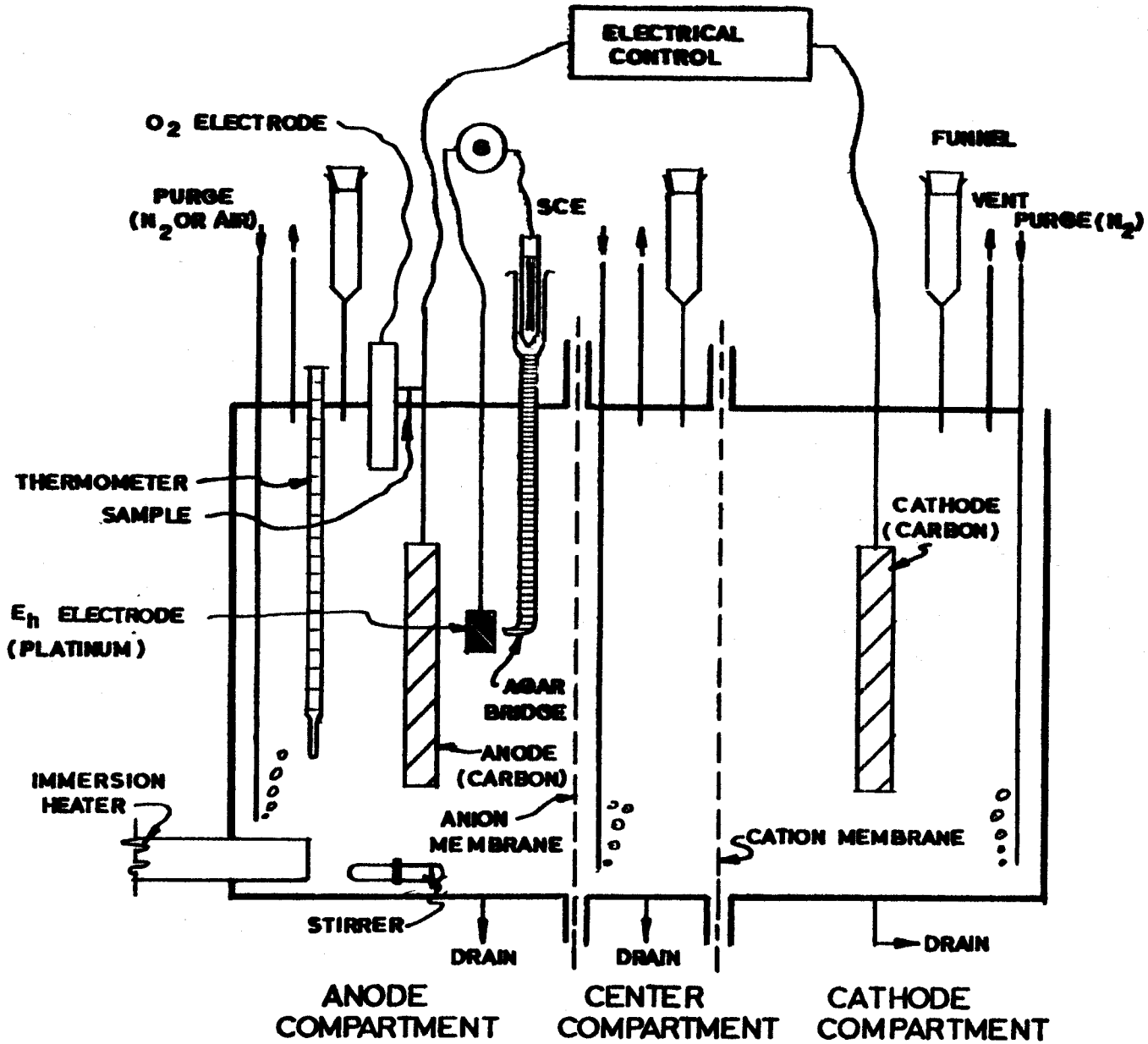


FIGURE II

Cell for Electrochemical Control of E_h

5. CONCLUSIONS

The maximum energy available from human wastes is approximately 74 watt-hours per man-day's output, 64% of which is derived from urea in urine. These figures are based upon (1) converting urine and fecal nitrogen to ammonia, (2) converting fecal polysaccharides to hydrogen, and (3) using these as electrochemical fuels with an oxygen cathode.

The literature search indicated that the conditions required for efficient utilization of urine and feces as electrochemical fuels are not compatible with those for waste purification. Thus, the system was optimized for conversion of urine and feces to electroactive chemicals since waste-treatment processes were a secondary consideration in these studies. Bacteria and enzymes were selected for study which would lead to the production of ammonia from urine and hydrogen from feces under anaerobic conditions.

Bacillus pasteurii was the most attractive ammonia-producing microorganism examined because of its relatively rapid anaerobic growth in urine accompanied by efficient urea hydrolysis. In continuous culture, approximately 0.5 mg ammonia was produced per ml of culture per hour during steady state conditions; 84% of the urea in urine was utilized.

In urine containing lyophilized feces (1 and 5%), ureolysis of B. pasteurii was not enhanced; with 10% feces in urine, inhibition was observed. Therefore, urine was separated from feces for use as a bioelectrochemical fuel.

Poor electrochemical performance was observed with B. pasteurii in urine. This is attributed to poisoning of the platinized platinum anode by ammonia.

Unaltered feces is not a suitable substrate for the production of hydrogen with a mixed sewage culture or a pure culture of Escherichia coli. The enzymes, cellulase and lipase, appear to be the best candidates for degrading complex fecal components to similar compounds in order to facilitate subsequent microbial activity.

6. RECOMMENDATIONS

The problem of disposal of wastes during extended manned space missions requires much more attention to arrive at an optimum solution. In the interest of trying to further close the cycle and provide greater self-sufficiency, methods and means of recovering as much useful material as possible with the least penalty from wastes must be pursued. Microbial waste processing is one such approach. Anaerobic and aerobic digestion methods have been considered. The anaerobic process is of interest primarily because the products could be used to provide some electrical energy in a fuel cell. It appears, however, that the amount of energy available from this source is too small, and the other gaseous by-products too difficult to handle to make anaerobic digestion attractive.

Aerobic digestion ("stabilization") is more rapid and efficient than anaerobic digestion, and produces a minimum of undesirable volatile by-products. This process is presently being studied for use in spacecraft. An analysis of aerobic bacterial digestion for use in space missions has raised the following points:

1. Assuming that the microbes are to be suspended in the medium and wastes are to be treated continuously, the subsystem must operate as a "chemostat," i.e., in such a way that the activity and population of microbes at any point in the system remains constant with time. Our experience with chemostat work indicates that this is very difficult to do. Activity tends to become too low or too high in spots. Batch processing might avoid the need for this degree of control, but would lead to other problems.

2. If the microbes are in suspension, a large quantity of both live and dead bacteria will come along with the output of the process, and must be removed from the liquid and disposed. This disposal problem may not be any simpler than that associated with the original waste materials.

3. Oxygen must be supplied in excess to the microbial suspension. The solubility of oxygen in water is rather low and thus, unless high pressures are used, excess oxygen is provided by means of a profusion of bubbles of undissolved gas throughout the medium. In a zero-gravity field, this gas phase, once dispersed in the liquid phase, would be most difficult to separate, and hence would tend to interfere seriously with further handling of the digested material.

In view of these considerations, we suggest instead that the process be carried out by contacting the waste stream with a fixed bed of appropriate microbes, and that the required oxygen be supplied electrolytically from the surface of the supporting structure. This bioelectrochemical approach should have the following advantages:

1. The activity of the microbes would be much easier to maintain constant, as growth on a solid surface tends to be self-regulating.

2. The amount of microbial matter passing out of the system with the liquid stream may be kept very small, due to the fact that a surface-growth tends to retain and utilize dead cells. This action is partially responsible for the self-regulation mentioned above.

3. No undissolved gas bubbles need appear in the bulk of the liquid phase. Oxygen generated electrochemically under the microbial layer would be consumed before an excess is produced. This would result in a net saving on power required to generate oxygen.

To avoid explosive mixtures of hydrogen and oxygen, a cathode on which one or more species of hydrogen-consuming bacteria are metabolizing can be used. These bacteria consume hydrogen, along with carbon dioxide and oxygen, to derive energy and build cell material. By exposing the bacterial layer to the aerobic digestion process stream, an adequate supply of CO_2 and O_2 will be provided.

No trouble is anticipated in selecting microorganisms capable of decomposing wastes and at the same time of adhering naturally to a solid surface. Many such species

are slime-formers and/or filamentous types which prefer to grow other than in suspension.

An extension and refinement of the fixed bed approach to microbiological waste handling is that of an immobilized enzyme preparation. In such a device the enzyme activity is tied down to a fixed matrix and the liquid stream flows past and is acted on in the process. The enzyme is thereby retained in a known location in the system.

Enzymes have some inherent advantages over whole cells for biochemical processing. They can be selected to accomplish specific reactions, rather than having to depend on the over-all metabolic process of a living organism. Also, the reaction rates achievable with enzymes are much higher than those observed with whole cells, provided conditions are optimized for each activity.

It is becoming clear that nitrogen (as N_2) may be an indispensible component of space cabin atmospheres for extended missions. As some leakage and adsorption will occur, nitrogen will gradually become depleted unless specific measures are taken to replace losses. Obviously, tanks of compressed nitrogen can be carried with their attendant weight penalty. Instead, however, we suggest that a bioelectrochemical waste digestion process might be so operated that an amount of N_2 sufficient for make-up is continuously generated, and at the same time ammonia is removed from the system.

Wherever microorganisms are used purposefully they must be monitored and controlled to insure stable, reliable performance. For this purpose, a rate of response from the sensor that is rapid relative to the maximum rate of change of microbial activity expected from the system is needed. A device having the necessary response characteristics and also suitable for use in space vehicles can be developed using a device under study at Magna. This is an electrochemical device that generates an electrical output proportional to redox enzyme activity in the vicinity of the sensing electrodes. The enzymes detected can be as part of either cell-free extracts or whole, living cells of microorganisms. As few as 10^4 bacteria per ml have been counted in 5 minutes using this technique. Greater populations can be counted in correspondingly

less time (the population in truly active cultures often ranges from 10^6 to 10^9 per ml).

Most work on this device to date has been applied to use as a detector for BW agents, where very low populations were of interest. We believe that the method can be adapted even more readily to measurement of higher populations expected to be encountered in microbial waste processing systems in space.

7. REFERENCES

1. Zubrzycki, L. and E. H. Spaulding, J. Bacteriol., 83, 968 (1962)

2. Smith, H. W. and W. E. Crabb, J. Pathol. Bacteriol., 82, 53 (1961)

3. Buthaux, R. and D. A. A. Mossel, J. Appl. Bacteriol., 24, 353 (1961)

4. Collee, L. G., J. A. Knowlden, and B. C. Hobbs, J. Appl. Bacteriol., 24, 326 (1961)

5. Brisou, B., Bull. Soc. Pathol. Exot., 54, 746 (1961)

6. Rosebury, T., Microorganisms Indigenous to Man, McGraw-Hill Book Company, Inc., New York (1962)

7. Goldblith, S. A. and E. L. Wick, Contract AF 33(616)-6136, ASO-TR-61-419 (1961)

8. Albritton, E. C., Standard Values in Nutrition and Metabolism, WADC Technical Report 52-301, Wright Air Development Center, Wright-Patterson Air Force Base, December 1953

9. Fantus, B., O. Wozasek, and S. Steigmann, Am. J. Digest. Dis., 8, 296 (1941)

10. Cantarow, A., and B. Schepartz, Biochemistry, W. B. Saunders Co., Philadelphia, Penn. (1954)

11. Tischer, R. G., B. P. Tischer and D. Cook, Developments in Industrial Microbiology. 3, 72, Plenum Press, N. Y., (1962)

12. Hawk, P. B., B. L. Oser and W. H. Summersen, Practical Physiological Chemistry, 12th Ed., 788, Lea and Febiger, Philadelphia, Penn. (1956)

13. Ingram, W. T., TDR #AMRL-TDR-62-126, Contract AF 33(616)-7827, (1962)

14. Butterfield, C., Publ. Health Repts., 50, 571 (1935)

15. Buck, T. C. and C. E. Keefer, Sew. and Ind. Wastes, 31, 1267 (1959)

16. McKinney, R. E. and R. G. Weichlein, Appl. Microbiol. 1, 259 (1953)

17. Kaplovsky, A. J., Appl. Microbiol., 5, 175 (1957)

18. Ruckhoft, C. C., J. G. Kallas and G. P. Edwards, J. Bacteriol., 19, 269 (1930)

19. Buck, T. C., C. E. Keefer and H. Hatch, Sew. and Ind. Wastes, 26, 164 (1954)

20. Bergey's Manual of Determinative Bacteriology, Seventh Edition, The Williams and Williams Co., Baltimore, Md. (1957)

21. Leone, D. E., Contract NASw-95, U413-63-041, General Dynamics, Electric Boat Division, Groton, Conn. (1963)

22. Kountz, R. R. and C. Forney, Jr., Sew. and Ind. Waste, 31, 819 (1959)

23. Garrett, M. T. Jr., Sew. and Ind. Waste, 30, 253 (1958)

24. Golueke, C. G., W. J. Oswald and P. H. McGanky, Sew. and Ind. Waste, 31, 1125 (1959)

25. Unger, M., NASA Lewis, Personal Communication (1964)

26. Moyer, J. E., TDR No. AMRL-TDR-62-116, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio, p. 281, (1962)

27. Pote, H. L., TDR No. AMRL-TDR-62-116, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio, p. 290 (1962)

28. Wheaton, R. B., J. J. Symons, N. G. Roth and H. H. Morris, TDR. No. AMRL-TDR-62-116, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio, p. 295 (1962)

29. Melpar Corp., Contract DA 36-039 SC-90878, Second Quarterly Progress Report, 1 Oct. - 31 Dec. 1962

30. Melpar Corp., Contract DA 36-039 SC-90878, Fifth Quarterly Progress Report, 1 July - 30 Sept. 1963

31. Gest, H., Bacteriol. Revs., 18, 43 (1954)

32. Stickland, L. H., Bioch. J., 28, 1746 (1934)

33. Nisman, R., M. Raynard, and G. N. Cohen, Arch. Biochem., 16, 473 (1948)

34. Khouvine, Y., Ann. de l' Inst. Past., 37, 711 (1923)

35. Cowles, P. B. and L. F. Rettger, J. Bacteriol., 21, 167 (1931)

36. Nagliski, J., J. W. White, Jr., S. R. Hoover and J. J. Willamin, J. Bacteriol., 49, 563 (1945)

37. Hungate, R. E., Bact. Revs., 14, 1 (1950)

38. Fuller, W. H. and A. G. Norman, J. Bacteriol., 46, 273 (1943)

39. Fuller, W. H. and A. G. Norman, J. Bacteriol., 46, 281 (1943)

40. Fuller, W. H. and A. G. Norman, J. Bacteriol., 46, 291 (1943)

41. Alexander, M., Introduction to Soil Microbiology, John Wiley and Sons, Inc., N. Y. (1961)

42. Wiley, W. H. and J. R. Stokes, J. Bacteriol., 84, 730 (1962)

43. Gibbons, R. J. and R. N. Doetsch, J. Bacteriol., 77, 417 (1959)

44. Cooke, J. V. and H. R. Keith, J. Bacteriol., 13, 315 (1927)

45. Holo and Szilagyi, Ind. agr. et. aliment. (Paris) 74, 131 (1957)

46. Greenfield and Lane, J. Biol. Chem., 204, 669 (1953)

47. Hungate, R. E., Biol. Bull., 83, 303 (1942)

48. Misra and Ranganathan, Proc. Ind. Acad. Sci., 39B, 100 (1954)

49. Enebo et al., J. of the Inst. of Brew., 59, 207 (1953)

50. Duncan, Manners and Ross, Biochem. J., 63, 44 (1956)

51. Whitaker, Arch. Biochem. Biophys., 43, 253 (1953)

52. Stone, Biochem. J., 66, 1 (1957)

53. Festenstein, Biochem. J., 69, 562 (1958)

54. Okamoto and Asai, J. Agric. Chem. Soc. (Japan), 26 (1952)

55. Nisizawa, J. Biochem. (Japan) 42, 825 (1955)

56. Seillere, C. R., Soc. Bio. (Paris), 61, 205 (1906)

57. Seillere, C. R., Soc. Bio. (Paris), 63, 151 (1907)

58. Seillere, C. R., Soc. Bio. (Paris), 68, 107 (1910)

59. Karrer and Illing, Kolloidzschr., 36, 91 (1925)

60. Husemann and Lotterle, Makromol. Chem., 4, 278 (1950)

61. Gascoigne, J. A. and M. M. Casoigne, Biological Degradation of Cellulose, Butterworths, London, England (1960)

62. Saunders, Sire and Genest, J. Biol. Chem., 174, 697 (1948)

63. Sison, Schubert and Nord, Arch. Biochem. Biophys., 75, 260 (1958)

64. Koviman, Enzymologia, 18, 22 (1957)

65. Studies in Cellulose Decomposition, Enebo, Stockholm (1954)

66. Toyama, J. Ferment. Technol. (Japan), 34, 281 (1956)

67. Stone, Ph. D. Thesis, University of London (1954)

68. Jermyn, Aust. J. Sci. Res., B5, 409 (1952)

69. Basu and Whitaker, Arch. Biochem. Biophys., 42, 12 (1953)

70. Reese, Gilligan and Norkraus, Physiol. Plant, 5, 379 (1952)

71. Whitaker, Science, 116, 90 (1952)

72. Lineweaver, H., and G. A. Ballow, Arch. Biochem., 6, 373 (1945)

73. Pithawala, R. R., C. R. Savur and A. Screenivasan, Arch. Biochem., 17, 235 (1948)

74. Owens, H. S., R. M. McCready and W. D. Maclay, Ind. Eng. Chem., 36, 936 (1944)

75. Holden, M., Biochem. J., 40, 103 (1946)

76. McColloch, R. J. and Z. I. Kertesz, Arch. Biochem., 13, 217 (1947)

77. Deuel H. and E. Stutz, Advances in Enzymology, Vol. 20, Interscience Publishers, Inc., N. Y., edited by F. F. Nord (1958)

78. Schultz, T. H., H. Lotzkar, H. S. Owens and W. D. Maclay, J. Phys. Chem., 49, 554 (1945)

79. MacDonnell, L. R., E. F. Jansen and H. Lineweaver, Arch. Biochem., 6, 389 (1945)

80. Lineweaver, H. and E. F. Jansen, in Advances in Enzymology, Vol II, Interscience Publishers, Inc., N. Y., edited by F. F. Nord (1951)

81. McColloch, R. J., J. C. Moyer and Z. I. Kertesz, Arch. Biochem., 10, 479 (1946)

82. Mills, C. B., Biochem. J., 44, 302 (1949)

83. Schubert, E., Biochem. J., 78, 323 (1952)

84. Ozawa, J., Ber. Ohara Inst. landwirtsch. Forsch. Kuraskiki Japan, 9, 431, (1951)

85. Lineweaver, H., R. Jang and E. F. Jansen, Arch. Biochem., 20, 131 (1949)

86. Pallmann, H. and H. Deuel, H. Chimia, 1, 27 (1947)

87. Jansen, E. F., L. R. MacDonnell and R. Jang, Arch. Biochem., 8, 113 (1945)

88. Schubert, E., Melliand Textilber, 8, 1 (1954)

89. Koch, J., Fruchtsaft-Ind., 1, 66 (1956)

90. Jansen, E. F., R. Jang, L. R. MacDonnell, Arch. Biochem., 15, 415 (1947)

91. Deuel, H., and F. Weber, Helv. Chim. Acta, 29, 1872 (1946)

92. Wills, E. D., Biochem. J., 69, 17 (1958)

93. Marchis-Monrew, G., L. Sarda and P. Desnuelle, Biochem. et. Biophys. Acta, 41, 358 (1960)

94. Biochemical Problems of Lipids, edited by B. Borgstromm, G. Popjak and E. LeBreton, Butterworths, London, England (1956)

95. Schonheyder, F. and K. Volqvartz, Enzymologia, 11, 178 (1944)

96. Fiore, J. V. and F. F. Nord, Arch. Biochem., 26, 382 (1950)

97. Gorbach, G. and H. Guntner, Sitzber. Akad. Wiss. Wein. Mathnaturw, Kl, Abt. IIb, 141, 415 (1932)

98. Connstein, W., E. Hoyer and H. von Wartenberg, Ber. 35, 3988 (1902)

99. Bayliss, M., D. Glick and R. A. Siem, J. Bacteriol., 55, 307 (1948)

100. Starr, M. P. and W. H. Burkholder, Phytopathology, 32, 598 (1942)

101. The Enzymes of Lipid Metabolism, edited by P. Desnuelle, Pergamon Press, London, England (1961)

102. Wills, E. D., Biochem. Biophys. Acta, 40, 487 (1950)

103. Weinsteine, S. S. and A. M. Wynne, J. Biol. Chem., 112, 641, 649 (1935-36)

104. Constantin, M. J., L. Pasero and P. Desnuelle, Biochem. et. Biophys. Acta, 43, 103 (1960)

105. Savory, P. and P. Desnuelle, Biochem. et Biophys. Acta, 21, 349 (1956)

106. Schonheyder, F. and K. Volquartz, Acta Physiol. Scand., 10, 62 (1945); 11, 349 (1946)

107. Methods in Enzymology, Vol I, edited by S. Colowick and N. Kaplan, Academic Press Inc., N. Y. (1955)

108. Wills, E. D., Biochem. J., 57, 109 (1954)

109. Kimmel, J. R. and E. L. Smith, J. Biol. Chem., 207, 515 (1954)

110. Hwang, K. and A. C. Evy, Ann. New York Acad. Sci., 54, 161 (1951)

111. Lineweaver, H. and S. Schwimmer, Enzymologia, 10, 81 (1941)

112. Hill, R. L., H. C. Schwartz and E. L. Smith, J. Biol. Chem., 234, 573 (1959)

113. Ball, A. K. and H. Lineweaver, J. Biol. Chem., 130, 669 (1939)

114. Kimmel, J. R. and E. L. Smith, J. Biol. Chem., 207, 515 (1954)

115. Calvery, H. O., J. Biol. Chem., 102, 73 (1933)

116. Methods in Enzymology, Vol. 2, edited by Sidney P. Colowick and Nathan O. Kaplan, Academic Press, Inc., N. Y. (1955)

117. Irving, G. W. Jr., J. S. Fruton and M. Bergmann, J. Biol. Chem., 138, 231 (1941)

118. Anson, M. L., J. Gen. Physiol., 22, 79 (1938)

119. Kunitzy, M., J. Gen. Physiol., 30, 311 (1947)

120. Larson, A. D. and R. E. Kallio, J. Bacteriol., 68, 67 (1954)

121. Dounce, A. L., J. Biol. Chem., 140, 307 (1941)

122. Sumner, J. B. and L. O. Poland, Proc. Soc. Exptl. Biol. Med., 30, 553, (1933)

123. Hellerman, L., F. B. Chinard and U. R. Dietz, J. Biol. Chem., 147, 443 (1943)

124. Fosman, G. D. and C. Niemann, J. Am. Chem. Soc., 73, 1646 (1951)

125. Wall, M. C. and K. J. Laidler, ABB, 43, 299 (1953)

126. Wall, M. C. and K. J. Laidler, ABB, 43, 307 (1953)

127. Laidler, K. J. and J. P. Hoarle, J. Am. Chem. Soc., 72, 2489 (1950)

128. Kistiakowsky, C. B. and R. Lumry, J. Am. Chem. Soc., 71, 2006 (1949)

129. The Enzymes, 1st Ed., Vol. I, edited by J. B. Sumner and K. Myrbäck, Academic Press, Inc., New York (1951)

130. Shaw, W. H. R. and C. B. Kistiakowsky, J. Am. Chem. Soc., 72, 2817 (1950)

131. Gorin, et al., Biochemistry, 1, 911 (1962)

132. Gentzkow, C. J., J. Biol. Chem., 143, 531 (1942)

133. Ormsby, A. A., J. Biol. Chem., 146, 595 (1942)

134. Bornside, G. A. and R. E. Kallio, J. Bacteriol., 71, 627 (1955)

135. Herbert, D., Symposia Intern. Congr. Microbiol., 7th Congr., Stockholm (1958)

136. Bryson, V., Science, 116, 48 (1952)

137. Novick, A., Science, 112, 715 (1950)

138. Monod, J., Ann. Inst. Pasteur, 79, 390 (1950)

139. Iyerusalimski, N. D., Izvestia Akademii Naub. SSSR Seriya, Biologicteskoya, Nr. 3, 418 (1962)

140. Sumner, J. B., J. Biol. Chem., 193, 265 (1951)

141. Finkelstein, B., Whirlpool Corp., St. Joseph, Mich., Personal Communication (1963)

142. Standard Methods for the Examination of Water and Wastewater, Eleventh Edition, American Public Health Association, N. Y., (1962)

143. Lowry, O. H., N. J. Rosenbrough, A. C. Farr and R. J. Randall, J. Biol. Chem., 193, 265 (1951)

8. IDENTIFICATION OF KEY PERSONNEL

Key technical personnel assigned to this contract are as follows:

	Man-hours
J. H. Canfield, Head, Life Sciences (Project Leader)	397
J. J. Cavallo, Research Biochemist	213
B. H. Goldner, Senior Research Microbiologist	849
M. D. Lechtman, Research Microbiologist	732
R. Lutwack, Senior Research Chemist	290
C. Albright, Research Chemist	1,056
J. Dittman, Research Microbiologist	538
Technicians	577